University of Alberta

Exploring novel strategies to prevent endotoxin-related diseases in periparturient dairy cows

by

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DEDICATION

This thesis is dedicated to my Aacharya (Teacher) ***Dr. Burim N. Ametaj*** *for supporting and believing in me all the way through. This dedication will not be complete if I do not mention my late father* ***Er. G. Sivaraman,*** *my mother* ***S. Saroja,*** *my sister* ***S. Premalatha,*** *my brother in law* ***L. Venkatesan,*** *and my wife* ***T. Suyambhiga,*** *for their dedicated support to me to finish this thesis and graduation. I have no words to express my excitement and inspiration I received from my just born daughter* ***Vairathi Lashmi*** *to complete my thesis and graduation. I also thank* ***Suzanna Dunn*** *for her continuous technical and moral support to finish my thesis. Additionally, I thank all my friends particularly my roommates Sandeep and Dileep for helping and encouraging me to finish my graduation and thesis. Finally, I will never forget* ***Ian Blackenberg*** *(Residence coordinator) and my colleagues and staff at Patient Food Service, University of Alberta Hospital especially* ***Andrea Littlejohn, Barbara Marocco,*** *and* ***Fanny Biasini*** *who stood by me during my hard times.*

ABSTRACT

Thirty two pregnant Holstein dairy cows were assigned to 4 treatment groups 2 weeks before the expected day of parturition. Cows were administered orally or intravenously, twice per week, for 3 consecutive weeks starting 2 weeks before parturition saline, or saline containing LPS from *Escherichia coli* 0111:B4. The amount of LPS administered each week was 0.01, 0.05, or 0.1 μg/kg BW. Blood samples were obtained from the jugular vein twice per week for 3 consecutive weeks 2 wk before and 1 wk after calving as well as once per week on wk 2, 3, 4 after calving. Plasma serum Amyloid A, lipopolysaccharide binding protein, haptoglobin, immunoglobulin A, immunoglobulin G, immunoglobulin M, cortisol, non-esterified fatty acids, beta-hydroxy butyric acid, glucose, lactate, insulin cholesterol, calcium, iron, zinc, and copper were measured. Taken together results of this study indicate that repeated oral administration of LPS during peripartum improved immune status and prevented clinical and metabolite disturbances typically observed in dairy cows around parturition. On the other hand, intravenous infusion of LPS increased incidence of metabolic and infectious diseases in periparturient dairy cows.

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LIST OF ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
APC	Antigen-presenting cell
APP	Acute phase proteins
APR	Acute phase response
ARA	Acute ruminal acidosis
BHBA	Beta-hydroxy butyric acid
Ca^{2+}	Calcium
CD	Cluster differentiation
CTL	Cytotoxic T lymphocyte
Cu^{2+}	Copper
DC	Dendritic cell
DIM	Days in milk
DMI	Dry matter intake
ELISA	Enzyme-linked immunosorbent assay
Fe^{2+}	Iron
HDL	High-density lipoproteins
HPA	Hepatic-pituitary-adrenal
Hp	Haptoglobin
HEK	Human embryonic kidney cells
IFN	Interferon
IL	Interleukin
IRAK	Interleukin receptor associated kinase
JNK	c-Jun N-terminal kinase
KDO	2-keto-2-deoxyoctonate
LBP	Lipopolysaccharide binding protein
LDL	Low-density lipoproteins
LPS	Lipopolysaccharide
MALT	Mucosa-associated lymphoid tissues
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
MT	Metallothionine
NEB	Negative energy balance
NEFA	Non-esterified fatty acids
NF-κB	Nuclear factor-κB
NK	Natural killer cells
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PGE_2	Prostaglandin E_2
PRR	Pattern recognition receptors
PTH	Parathyroid hormone
SAA	Serum amyloid A
SAP	Stress-activated protein kinase
SARA	Subacute ruminal acidosis

SEM	Standard error of mean
SIgA	Secretory IgA
TAG	Triacylglycerols
TGF	Transforming growth factor
TLR	Toll-like receptors
TMR	Total mixed ration
TNF	Tumor necrosis factor
TRAF	Tumor necrosis factor receptor associated factor
VLDL	Very low-density lipoprotein
Zn^{2+}	Zinc

Chapter 1

General Introduction

Transition period including 3 weeks before and 3 weeks after parturition is characterized by major changes in a cow's physiology and metabolism. Initiation of lactation requires large amounts of energy and there is a 30% decline in feed intake (Grant and Alright, 1995). Because the energy input is lower than the energy output a negative energy balance develops and cows start mobilizing fatty acids from adipose tissue to provide the necessary energy needed for milk synthesis (Grummer et al., 1995). Another important change during the transition period is initiation of a new ration containing high proportions of grain. Feeding high grain to ruminants is associated with development of a state of subacute acidosis (Ametaj et al., 2005) as well as a variety of interconnected metabolic diseases like laminitis, displaced abomasum, fatty liver, liver abscesses or bloat (Bevans et al., 2005; Brent, 1976; Brink et al., 1990; Goad et al., 1998). The reason for high association of grain with multiple metabolic diseases is not very clear; however, there is an increasing body of evidence pointing out the importance of endotoxin in the etiology and pathogenesis of multiple metabolic diseases (Emmanuel et al., 2008).

Research conducted by Dr. Ametaj's team at University of Alberta has shown that feeding increasing proportions of barley grain is associated with the release of large amounts of endotoxin in the rumen fluid (Emmanuel et al., 2008). Additionally, we also demonstrated, under Ussing chamber conditions, that endotoxin permeates easily through rumen and colon samples at concentrations very similar to that found in the rumen fluid of ruminants fed high amounts of grain (Emmanuel et al., 2007). Translocation of

endotoxin into the blood circulation of the host has been shown to be associated with multiple metabolic, hormonal, immunologic, and health changes. Different strategies have been employed in the past to prevent or treat negative effects of endotoxin. Some investigators have used intravenous infusion of immunoglobulins as a means to eliminate the translocated endotoxin from host circulation. In addition, other investigators have used intravenous infusion of reconstituted high-density lipoproteins to bind and neutralize endotoxin present in blood. Moreover, there are many reports related to infusion of lipopolysaccharide (LPS) in blood to induce production of anti-LPS antibodies and develop tolerance to endotoxin (Alan, 2002). Unfortunately, most of the aforementioned preventive or treatment interventions have not been very successful and endotoxin continues to be a major causal agent of a variety of diseases in humans and animals alike.

Since the major source of endotoxin are Gram-negative bacteria living in the gastrointestinal tract or other mucosal layers like mammary gland or reproductive tract it would be of interest to develop a mucosal immune response against endotoxin to prevent its translocation to the host blood or lymphatic circulation. Based on a new hypothesis developed by Dr. Ametaj, we tested the postulate that repeated oral application of LPS two weeks before and one week after parturition would be able to prevent transferring of endotoxin from mucosal layers into the host systemic circulation and metabolic diseases related to endotoxin. Two other hypotheses that we wanted to test were whether intravenous infusion of LPS would induce tolerance to endotoxin and prevent or deteriorate metabolic diseases and improve or worsen the general health status and productivity of dairy cows.

Results presented in this thesis show that oral administration of LPS from *Escherichia coli* 0111:B4 prevented metabolic disease and improved metabolic state and immune responses of transition dairy cows. On the other hand, intravenous infusion of LPS increased the incidence of metabolic diseases and was associated with perturbation of metabolism and immune responses.

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Chapter 2

Literature Review

2.1 Endotoxin-related diseases in dairy cows

Immediately after parturition dairy cows are affected by one or multiple disorders known as metabolic diseases including acidosis, laminitis, displacement of abomasum, fatty liver, ketosis, milk fever, downer cow syndrome, retained placenta, infertility, bloat, liver abscesses, mastitis, unexplained fever, and metritis. Interestingly, these diseases are though to be interrelated with each other (Erb et al., 1985); however, the reason why they associate with each other is not correctly understood. Different investigators have suggested various explanations for the interrelationship of metabolic diseases. For example, in a review by Grummer et al. (1993) the author classifies diseases such as fatty liver and ketosis as lipid-related disorders. Another investigator (Katoh, 2002) groups fatty liver, ketosis, left displacement of abomasum, downer cow syndrome, retained placenta, and mastitis as diseases related to disturbances of several proteins produced by the liver known as apolipoprotein (apo) A-I, apoB-48, apoB-100, and apoC-III as well as acute phase proteins such as serum amyloid A (SAA) and haptoglobin. However, the author gives no explanation to the reason why all these proteins are disturbed in cows around parturition. On the other hand, a group of other researchers has related metabolic diseases of post parturient cows to grain feeding and endotoxin released in the rumen during feeding of high-grain diets or in infected organs such as udder or mammary gland (Nagaraja et al., 1978; Andersen et al., 2003; Emmanuel et al., 2008). Andersen (2003) suggests that diseases such as acidosis, laminitis, displaced abomasum, and sudden death

syndrome are related to endotoxemia. Several other investigators support this hypothesis. For example, Ametaj et al. (2005) showed evidence that cows suffering from fatty liver have an inflammatory response present in their blood characterized by high plasma cytokines and acute phase proteins such as TNF-alpha, SAA, and haptoglobin. Furthermore, Aiumlamai et al. (1992) suggested a role for endotoxin in the etiology of milk fever. Ametaj et al. (2001) supported the latter hypothesis by demonstrating that dairy cows affected by milk fever have higher plasma acute phase proteins compared with normal cows. In addition, Fuerll et al. (2002) demonstrated high plasma endotoxin in cows suffering from milk fever. Aiumlamai et al. (1992) also suggested a role for endotoxin in the pathogenesis of ruminal acidosis. Recently, several other investigators bring evidence for a role of endotoxin in ruminal acidosis (Andersen et al., 1994; Gozho et al., 2005, 2006, 2007; Emmanuel et al., 2008). Endotoxin has also been implicated in poor reproductive performance and infertility. Kindahl et al. (1992) indicated that infected uteri absorbed endotoxin and Mateus et al. (2003) showed a relationship between uterine infection, endotoxin translocation in plasma, and resumption of postpartum ovarian activity in dairy cows. Dohmen et al. (2000) also showed high endotoxin concentrations in the uterine lochia in cows with retained placenta. Furthermore, Boosman et al. (1991) provided evidence for a role of endotoxin in the pathogenesis of acute laminitis in dairy cows. Lipopolysaccharide has been found to affect the contraction of smooth muscles from bovine abomasal antrum indicating that endotoxin also might be involved in the etiology of displaced abomasum in cattle (Kaze et al., 2004). In conclusion, increasing evidence suggest a causal role for endotoxin in multiple metabolic

disorders in dairy cows around parturition. However, no direct evidence has been provided yet for a role of endotoxin in the etiology of metabolic diseases in dairy cows.

2.2 What is endotoxin?

Mucosal layers of gastrointestinal tract, lungs, reproductive organ, and mammary gland are exposed to a large number of microorganisms (Van Soest, 1984; Sheldon and Dobson, 2004). Most of these bacteria are commensals, living in symbiosis with the host; however, some of them are pathogenic bacteria. Among the pathogenic bacteria, there are two groups of bacteria inhabiting the GI tract: Gram-negative and Gram-positive bacteria. Many species of Gram-negative bacteria are pathogenic, meaning they can cause disease in a host organism. This pathogenic capability is often associated with certain components of Gram-negative cell walls, in particular the lipopolysaccharide (also known as LPS or endotoxin) layer. In fact, Gram-negative bacteria have a double outer membrane (Feulner et al., 2003; Figure. 2.1). The outer membrane is unique in its asymmetry. The inner leaflet of the outer membrane is composed of the same phospholipids as in the cytoplasmic membrane of other mammalian cells such as phosphatidylglycerol (PG), phosphatidylethanolamine (PE), and cardiolipin (Feulner et al., 2004). The outer leaflet contains predominantly a lipid component known as LPS. Lipopolysaccharide is located on the outer face of the membrane, where it mediates contact with the environment.

2.3 Functions of LPS in Gram negative bacteria

Lipopolysaccharide is essential as a structural component of the bacterial cell and has several functions. First, it serves as a permeability barrier that allows only low molecular weight, hydrophilic molecules to permeate through the membrane. Second, it impedes destruction of bacterial cells by plasma components and phagocytic cells. Third, LPS plays an important role as a surface structure in the interaction of the pathogen with its host. For example, LPS may be involved in adherence (colonization), or resistance to phagocytosis, or antigenic shifts that determine the course and outcome of an infection. Lipopolysaccharide also is a constituent of outer vesicles that are used by Gram-negative bacteria to kill competing microorganisms (Li et al., 1996). Therefore, presence of LPS in bacteria favors bacterial persistence on host mucosa, whereas recognizing it allows the host to kill invading bacteria within subepithelial tissues and prevent dissemination.

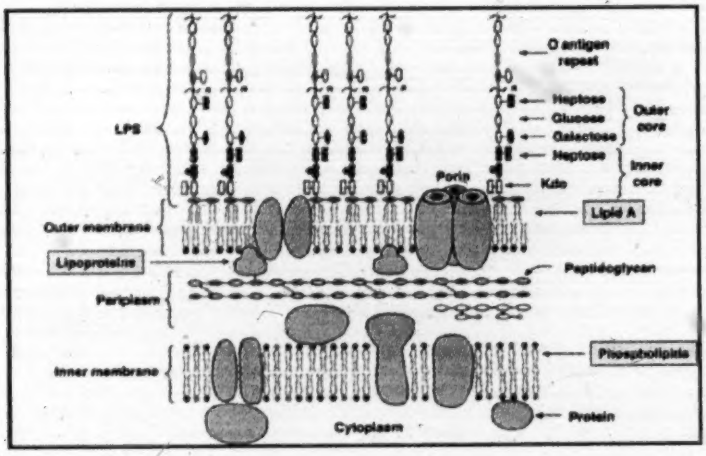


Figure 2.1. The composition of a Gram-negative bacterial membrane. The inner membrane surrounds the bacterial cell. The outer membrane surrounds the periplasm, which contains peptidoglycan. Lipopolysaccharide (LPS) is embedded in the outer leaflet of the outer membrane and is composed of three distinct components; lipid A,

oligosaccharide core, and O-antigen. The oligosaccharide core contains a unique sugar, 2-keto-2-deoxyoctonate (KDO). (Figure adopted from Feulner, 2003).

2.4 Structure of LPS

As the name indicates, LPS consists of a lipid region, termed lipid A attached to a polysaccharide region (Feulner, 2003, Figure 2.2). The polysaccharide region is composed of three separate components; an inner-core, an outer core, and the O-specific chain or O-polysaccharide. Each component has distinct structural and functional properties. The lipid A component of LPS anchors the molecule to the cell surface by insertion into the outer membrane. The lipid A moeity is linked to the polysaccharide region through a ketosidic linkage. The core oligosaccharide is composed of a short series of sugars and is divided into two domains, the O-polysaccharide-proximal outer core and the lipid A proximal inner core (Feulner et al., 2004). Presence of unusual sugars such as heptose (Hep) and Kdo characterize the inner core region. Linking the reducing end of the inner core region with lipid A is a α-ketosidic Kdo residue, termed Kdo I. Kdo I is further substituted by a α-linked Kdo [Kdo II], which in turn may be non-stoichiometrically substituted by another Kdo [Kdo III] (Feulner et al., 2003, 2004). A Hep residue is usually attached to Kdo I and, this may be substituted by another Hep residue to which the outer core region is attached. The inner core region may also have other monosaccharides or charged entities such as phosphate and ethanolamine. The inner core may play a role in modulating lipid A activity and in the induction of cytokines such as interleukin (IL)-1 or leukotriene (Feulner et al., 2003, 2004). Furthermore, this region represents the high affinity-binding site for divalent cations of importance in maintaining outer membrane integrity of some bacterial species.

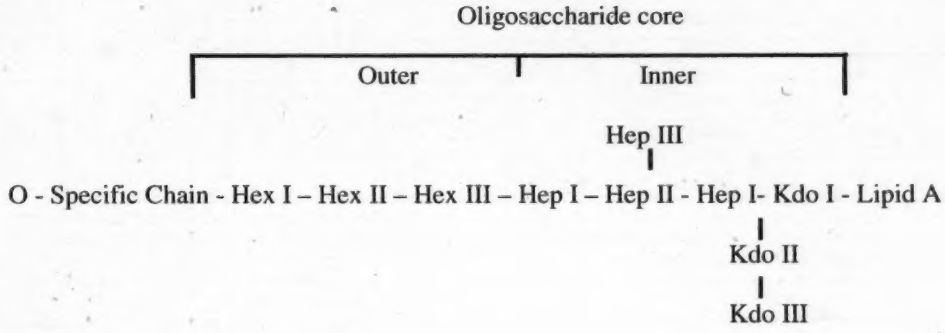


Figure 2.2. General structure of smooth LPS showing the O-specific chain, inner and outer core, and lipid A. (KDO =2-keto-2-deoxyoctonate; Hep = heptose; Hex = hexose). (Figure adopted from Feulner et al., 2003).

To recognize the lipid A moiety of the cell wall LPS on Gram-negative bacteria, animals use toll-like receptors (TLR) and the pattern recognition receptors (PRRs) or CD14–MD-2–TLR4 receptor mechanism (Janssens and Beyaert, 2003).

2.5 Sources of endotoxin in dairy cows

Ruminants are animals that have evolved with the ability to digest mainly forage diets. In the last few decades, the consumer demand for milk and dairy products has increased considerably (AAFC, 2008). The average milk production per cow has increased in the past 30 years in North America, finding annual productions up to 12,000 kg per animal (Tamminga et al., 2000, Casalmiglia, et al., 2006). In this regard, the use of total mixed rations (TMR) has been favoured as well as the inclusion of concentrates, in order to satisfy milk quotas and to take advantage of subsidies in grains such as maize, barley and oats (Maltz et al., 1992. To compensate for this demand, the dietary rations provided to dairy cows were designed to support high-milk production. Such diets were manipulated

drastically to provide high energy in a small volume of feedstuff. Therefore, grains were selected as the feed of choice to provide a diet high in energy.

Transition period, which involves 3 weeks before and 3 weeks after parturition is characterized by three stressful events including parturition, initiation of milk production, and a major change in the diet provided to dairy cows (Goff and Horst, 1997). During the first week after parturition, dairy cows in North America are switched from a diet rich in hay and roughage during the dry period to a diet very rich in grain immediately after parturition. This leads to several major changes in the digestive tract of ruminants including: 1) a rapid decline in ruminal pH, 2) major changes in the microbial ecology as well as 3) the release of large amounts of endotoxin (Emmanuel et al., 2008).

Recently, Emmanuel et al. (2008) showed that feeding dairy cows increasing amounts of grain was associated with low ruminal pH. Thus, feeding dairy cows a diet containing 45% barley grain (DM basis) was associated with a rumen pH below 6.0, 8-12 h after the morning feeding. Research conducted by Russell et al. (2001) demonstrated major changes in the ruminal and gastrointestinal microflora composition during feeding of diets rich in grain. Thus, feeding high grain diets implicates ruminal acidosis and release of endotoxin by gram-negative bacteria when the pH falls beyond 6.0.

For example, concentration of free endotoxin in ruminal fluid was almost 20-fold higher in steers fed high-grain diet compared to steers fed only hay (Nagaraja et al., 1978). Moreover, Dougherty et al. (1975) demonstrated presence of endotoxin in the blood of

cattle after induced grain engorgement. Furthermore, Anderson et al. (1994) showed that induction of acidosis by overfeeding of grain in the diet was associated with increased concentration of endotoxin in the portal and arterial blood of dairy cows. In another study, Anderson et al. (1993) demonstrated that in cows fed high amounts of grain concentration of endotoxin in the rumen increased.

2.6. Translocation of endotoxin into the blood circulation

During the years a number of studies have attempted to clarify the mechanism by which endotoxin is transferred from gastrointestinal tract into the host's circulation. Some investigators have suggested that creation of an acidic rumen environment, changes in osmotic pressure as well as ruminal endotoxin resulting from feeding easily fermentable carbohydrates may render the rumen epithelium susceptible to injury and contribute to translocation of rumen endotoxin into the prehepatic bloodstream (Brent, 1976; Enemark et al., 2002; Kleen et al., 2003). Feeding high-grain diets to calves resulted in microlesions of ruminal papillae in sheep (Kay et al., 1969; McManus et al., 1977), which may facilitate translocation of endotoxin into circulation. Presence of endotoxin in the blood was demonstrated in cattle and sheep after induced grain engorgement (Dougherty et al., 1975; Andersen et al., 1994). Emmanuel et al. (2007) showed that under acidic conditions and in presence of LPS rumen and colon walls become 'leaky' to non-nutritious compounds such as mannitol. The latter authors also showed that LPS permeates rumen and colon walls when present in high concentrations in the Ringer lactate solution (Emmanuel et al., 2008). Endotoxin from the gastro-intestinal tract has been suggested as a source of endotoxin. Studies involving normal and LPS-shocked

animals as well as ligated segments of the intestine into which the endotoxin was administered showed resorption of endotoxin from gastrointestinal tract (Truszczynski and Pilaszek, 1969). These studies show that endotoxin may translocate from gastrointestinal tract into the host blood circulation. Endotoxin also gains access to blood in a number of other conditions. For example, cows with naturally occurring acute coliform mastitis develop bacteremia, thus introducing endotoxin directly into the circulation (Wenz et al., 2001). Furthermore, endotoxin was detected in the plasma of cows with endometritis with plasma concentrations being highest between d 1 and 12 postpartum (Mateus et al., 2003).

It should be noted that only a few authors have provided evidence for presence of endotoxin into the host circulation, whereas, most others have not been able to do so (Gozho et al., 2005, 2006, 2007; Emmanuel et al., 2008). The main reasons for this are that: 1) endotoxin is removed very quickly from blood circulation, and 2) health status of the animal has been shown to affect half-life of endotoxin in the bloodstream. For example, Andersen et al. (1996) showed that clinically healthy Jersey cows cleared endotoxin from blood circulation within 30 minutes after intravenous infusion of 25 μg/kg BW of LPS from *Escherichia coli* 055:B5. The same authors indicated that cows affected spontaneously by hepatic lipidosis were not able to clean the said dose of LPS and one of those cows died shortly after the LPS infusion. Because of the described difficulties in measuring endotoxin directly in plasma, most authors in human and rodent research studies have used indirect methods to evaluate presence of endotoxin in plasma by measuring plasma cytokines like IL-1, IL-6, and TNF-α (Note: there are no

commercial kits available for bovine cytokines), whereas bovine researchers have used mostly acute phase proteins to do so (Ametaj et al., 2005; Emmanuel et al., 2008).

2.7. Host responses to lipopolysaccharide

2.7.1. Metabolic responses to LPS. Several studies involving administration of LPS in dairy cows have reported a variety of clinical and metabolic responses including increased body temperature and decreased appetite as well as alterations in carbohydrate, lipid, protein, and mineral metabolism (Klasing, 1988). Research conducted in other animal species also indicates that endotoxemia or inflammatory conditions are usually associated with multiple disturbances in intermediary metabolism, including increased lipogenesis (Guckian, 1973; Canonico et al., 1977), decreased plasma ketone bodies (Neufeld et al., 1976, Neufeld et al., 1980) as well as increased plasma cholesterol (Memon et al., 2008).

Recent investigations demonstrated that intravenous administration of LPS in dairy cows is associated with a biphasic response of plasma glucose, initial hyperglycemia, followed by hypoglycemia several hours later (Werling et al., 1996; Steiger et al., 1999; Lohuis et al., 1998). During the hyperglycemic phase after LPS challenge the release of glucose from liver increases (Naylor and Kronfeld, 1985). LPS-induced changes in carbohydrate metabolism are contributed by secretion of hormones such as adrenocorticotropic hormone (ACTH), cortisol, and activation of the sympathetic nervous system (Boosman et al., 1990; Nonogaki and Iguchi, 1997). The interaction of macrophages and LPS produces cytokines like IL-1 which has been shown to increase concentrations of

glucagons in plasma which in turn increases plasma glucose by glycogenolysis (Zenser and Powanda, 1977). Furthermore, hypoglycemia associated with translocation of endotoxin results from elevated plasma insulin due to increased glucose utilization and direct insulin-like actions of endotoxin (Filkins and Buchanan, 1977; Wolfe et al., 1977).

During intravenous administration of LPS there is a marked stimulation of lipolysis, reflected by an increase in the concentration of NEFA and glycerol in plasma (Steiger et al., 1999). Enhanced concentrations of TNF-alpha, IL-1, and IL-6 as well as of ACTH, cortisol, and catecholamine and enhanced activity of the sympathetic nervous system contribute to the lipolytic response to LPS (Boosman et al., 1990; Nonogaki and Iguchi, 1997). In addition, a decrease in the utilization of NEFA by the muscle tissue, contributes to the LPS-induced increase in plasma NEFA (Romanowsky et al., 1980). The increase in lipolysis might have either beneficial or detrimental effects on the host. The increase in lipolysis by depleting adipose stores could contribute to the potentially harmful cachexia that is observed during infection and inflammation (Feingold et al., 1992a). However, the increase in NEFA flux from adipose tissue to other tissues could provide a source of energy, which may be beneficial, especially during infection-induced anorexia. Additionally, the increased delivery of NEFA to the liver could contribute to the hyperlipidemia that accompanies infection and inflammation. Studies have suggested that this hyperlipidemia may be a beneficial response for the host. Specifically, experiments have demonstrated that lipoproteins bind and thereby protect the animal from the toxic effects of endotoxin (Ulevitch et al., 1979, 1981; Van Lenten et al., 1986). Furthermore,

endotoxin is bound to VLDL in the circulation of normal individuals suggesting that this detoxifying mechanism may be operating in the normal course of activity.

Interestingly, endotoxemia or inflammatory conditions are accompanied with marked changes in plasma ketone bodies (Nuefeld et al., 1976). Endotoxin, a product of Gram-negatives, has an antiketogenic effect in cattle. For example, Waldron et al. (2003) reported a decline in plasma BHBA following LPS administration in dairy cattle. Huhtanen et al. (1993) reported that the decrease in BHBA was primarily due to inhibition of hepatic ketogenesis. Cytokines like TNF-α and IL-1 as well as insulin mediates the LPS-induced inhibition of ketogenesis in cattle (Memon et al., 1992). Waldron et al. (2003) observed that when LPS was given intravenously to dairy cows there was a marked increase in the concentration of NEFA and a drop in the levels of circulating BHBA. The implications of these effects of endotoxin on health of dairy cattle are not quite understood; however, as previously suggested, endotoxin might be involved in development of fatty liver (Ametaj et al., 2005).

Increased rates of hepatic de novo cholesterol synthesis have been observed in several different animal models of infection and endotoxemia. Isolated hepatocytes from rats treated with pneumococci exhibit enhanced rates of [14C] acetate incorporation into fatty acids and cholesterol (Canonico et al., 1977). In vivo studies have shown increased hepatic fatty acid and cholesterol synthesis in *Escherichia coli*-treated rats (Lanza-Jacoby and Tabares, 1990). Vasconcelos et al. (1989) have also shown that during cecal ligation and puncture, another model for Gram-negative sepsis, there is increased hepatic

cholesterol synthesis and HMG-CoA reductase activity. Recent studies suggest that many of the effects of infection are caused by endotoxin and its mediators TNF and/or IL-1 (12-14). Previous studies have shown that TNF-alpha and IL-1 mimic the effects of infection and LPS on hepatic fatty acid synthesis and ketone body production (Beutler and Cerami, 1986; Feingold and Grunfeld, 1987; Feingold et al., 1991). In fact, the effect of LPS on serum cholesterol levels has been reported to be biphasic (Memon et al., 2008). The same authors reported that low doses of LPS produce a dose-dependent increase in serum cholesterol (21% and 41% increase, respectively) whereas higher doses of LPS do not affect concentration of cholesterol in plasma. The mechanism for the biphasic response is not clear, however, different doses of LPS have been shown to exert different effects on lipid metabolism; low doses of LPS stimulate triacyglycerols (TAG) production by the liver whereas high doses of LPS inhibit TAG clearance and both of these mechanisms contribute to the hypertriglyceridemia of infection (Feingold et al., 1992). It is likely that at lower doses the increase in plasma cholesterol levels produced by LPS is secondary to LPS-induced increase in hepatic cholesterol synthesis, whereas moderate to higher doses of LPS may activate other pathways of cholesterol metabolism such as increased lipoprotein clearance by increasing low density lipoprotein receptor levels, increased conversion of newly synthesized cholesterol into bile acids, or enhanced esterification and storage of cholesterol. Indeed, Ametaj et al. (2005) reported enhanced plasma cholesterol in dairy cows immediately after parturition accompanied by an increase in LDL, HDL, and serum lipids.

Intravenous administration of LPS results in hyperlactemia in most non-ruminant species (Mizock, 1995) and in sheep (Naylor and Kronfeld, 1985). Furthermore, administration of endotoxin in pregnant cows was accompanied by increased concentrations of lactate (Olszanecki and Chlopicki, 1999). Therefore, elevated concentrations of plasma lactate are indicative of endotoxemia in different species (Giri et al., 1990; Olszanecki and Chlopicki, 1999). The lactic acidemia observed during endotoxemia is related to increased anaerobic metabolism of glucose in skeletal muscle (Shackleford et al., 1986; James et al., 1996). Release of lactate in plasma increases hepatic uptake of lactate nd contributes to hepatic glucose production during the phase of endotoxemia when hepatic glycogen stores are depleted. Interestingly, Giri et al. (1990) reported that hyperlactemia occurred only when 2.5 μg/kg, but not 1.0 μg/kg of LPS was administered intravenously to pregnant, nonlactating cows. Waldron et al. (2003) also showed no effect of the lower-dose LPS administration on plasma lactate concentration. The latter study also demonstrated that LPS administration increased the capacity of liver slices to oxidize [1-^{14}C] L-lactate to CO_2 (Waldron et al. 2003).

It is well established that bacterial endotoxin is a potent activator of the hypothalamo-pituitary axis (HPA; Beishuizen et al., 2003). Although previous studies have shown that LPS exerts this effect principally by stimulating corticotropin-releasing hormone (CRH) secretion (Beishuizen et al., 2003), there is also evidence for CRH-independent effects (Elenkov et al., 1992; Schotanus et al., 1994). It appears that LPS may act on each tissue comprising the HPA axis, because there is also evidence for a pituitary-independent effect of LPS on the adrenal gland, with LPS causing increased corticosterone secretion in both

intact and hypophysectomized rats (Suzuki et al., 1986). More recently, it has been shown that LPS inhibits ACTH-stimulated corticosterone secretion by cultured rat zona glomerulosa cells, although the effect on basal secretion was not determined (Enrique de Salamanca and Garcia, 2003). Previous studies using human subjects *in vivo* have shown a two-stage cortisol response to LPS administration (Vedder et al., 1999). The first part of the response was seen about 3–4 h after LPS administration and coincided with the peak ACTH response. Both cortisol and ACTH returned to basal levels by 8 h, and cortisol, but not ACTH, then increased again, with a peak about 11–12 h after LPS administration. It seems likely that this second phase of the cortisol response may be due to a direct action of LPS on the adrenal cortex (Vakharia and Hinson, 2005). Research conducted in dairy cows and veal calves also has demonstrated that intravenous exposure to LPS produces a stress like condition manifested by the increase in plasma cortisol several hours after LPS administration (Hüsler and Blum, 2002; Waldron et al., 2003). Additionally, Hüsler and Blum (2002) reported that cortisol increased transiently in calves after LPS administration in agreement with Kenison et al. (1991) and Kinsbergen et al. (1994), which was likely and in part the consequence of the release of TNF-α (Klasing, 1988).

Minerals are essential components of body enzymes, hormones and cells. Minerals are required by dairy cows for optimum health and productivity, and are grouped as macrominerals and microminerals. Macrominerals are needed in greater amounts and are usually expressed as a percentage of the ration DM. Calcium, phosphorus, magnesium, and potassium are macrominerals that are important to the dairy cow. Microminerals are needed in smaller amounts and are generally expressed as parts per million (ppm). Iodine,

zinc, iron, copper, molybdenum, fluorine, cobalt, selenium, and chromium are microminerals needed by the dairy cow.

Calcium is the most abundant mineral in the body. Furthermore, blood calcium and phosphorus decreased after intravenous administration of LPS (Waldron et al., 2003). Approximately 98% of Ca^{2+} functions as a structural component of bones and teeth. The remaining 2% is distributed in extracellular fluids and soft tissues, and is involved in such vital functions as blood clotting, muscle contraction, nerve transmission, and as second messenger regulating the actions of many hormones (Horst, 1986). Hypocalcaemia increases the susceptibility of dairy cows to infectious disease and it has been documented that cows with milk fever are more likely to contract mastitis (Curtis et al., 1985). Hypocalcemia is observed and a marked decrease in concentration of Ca^{2+} in blood is seen within the first 2 h of LPS infusion in pigs (Carlstedt et al., 2000). Administration of LPS has been shown to inhibit the function of 1, 25-dihydroxy vitamin D_3 and lower secretion or action of PTH in patients with sepsis (Pramanik et al., 2004), however, there is no convincing evidence that these factors are responsible for hypocalcemia observed after LPS challenge.

Iron is an essential component of hemoglobin and myoglobin, the key proteins involved in oxygen transport and utilization. A number of mammalian enzymes like cytochromes and iron-sulphur proteins contain iron and are involved in the electron transport chain (McDowell, 1992). Approximately, two-thirds of body Fe^{2+} is present in hemoglobin in red blood cells and myoglobin in muscle. Infections with Gram-negative or Gram-

positive bacteria are associated with a decline in plasma Fe^{2+} as bacteria require Fe^{2+} for their growth and to exert their pathological effects (Bullen, 1981; Lauffer, 1992).

Zinc is one of the most important trace elements in the body and is known to participate in the activation of approximately 300 enzymes. Many enzymes, signaling molecules and transcription factors are critically dependent and regulated by Zn^{2+}. Zinc plays important roles in the immune response of the body. Lymphocyte functions such as mitogenesis, antibody synthesis, activation of T-cells, and natural killer cells as well as cellular immunity are regulated by Zn^{2+} (Antoniou et al., 1981; Moulder and Steward, 1989). Improper cellular immunity resulting from Zn^{2+} deficiency can predispose to bacterial, viral, and fungal infections. Thymic atrophy, anergic T-cells, decreased lymphocyte proliferative response, decreased T-helper cells, and reduced thymic hormone secretion are some of other disorders associated with Zn^{2+} deficiency (Falchuk, 1998; Wellinghausen and Rink, 1998).

Iron and Zn^{2+} are often limiting factors for infectious microbial growth (Neilands, 1981; Hantke, 2004). Iron serves as an important required component of hemoproteins that play important roles in metabolic reduction/oxidation reactions, respiration, and other critical cellular functions. Zinc serves as an important component of metalloproteases and other enzymes that protect against oxidative damage (Hase and Finkelstein, 1993; Schnell and Steinman, 1995). High serum iron concentrations can exacerbate infection by serving as an available nutrient source for pathogenic microbes (Weinberg, 1984, 1999). Higher vertebrates have evolved elaborate methods for withholding iron from invading microbes

while maintaining access to the metal themselves. Expression of iron-binding proteins such as lactoferrin, transferrin, and ferritin play important roles in iron metabolism and homeostasis during infection (Weinberg, 1984). These proteins help to sequester divalent metals away from microbes but still provide availability and access to the host organism. Iron withholding is an important innate defense mechanism in most of the higher vertebrates. This phenomenon has been identified in a broad diversity of phyla, including plants (Expert, 1999), insects (Nichol et al., 2002), birds (Giansanti et al., 2002), and mammals (Weinberg, 1999), and thus it has been deemed an ancient innate defense mechanism.

Hypozincemia is observed in response to endotoxemia or inflammation and this is related to redistribution of plasma Zn^{2+} to hepatocytes (Erskine and Barlett 1993). Proteins called metallothioneins (MT) are known to be involved in the Zn^{2+} homeostasis. Interleukin-6, which is proinflammatory and secreted in response to LPS, induces the production of metallothioneins (MT) in the liver resulting in the sequestration of Zn^{2+} into the liver hepatocytes (Brady et al., 1987). The effects of LPS on circulating levels of Zn^{2+} were studied in normal and MT-knockout mice. While normal mice exhibit gross hypozincemia, MT-knockout mice have normal concentrations of plasma Zn^{2+} indicating that MT is the principal factor responsible for the decline in plasma Zn^{2+} during acute phase response (APR; Rofe et al., 1996).

Copper functions as an essential component of a number of enzymes. Cu^{2+} is important for cell proliferation in the bone marrow; the earliest manifestations of Cu^{2+} deficiency

are anemia and leucopenia. Failure to meet the requirements for Cu^{2+} leads to a variety of clinical conditions like anemia, reduced growth, diarrhea and poor fertility (Underwood, 1981). Copper deficiency also reduces the phagocytic ability of isolated neutrophils and increases the susceptibility to bacterial infections (Woolliams et al., 1986). Poor availability of Cu^{2+} also leads to an impaired glucose and lipid metabolism and depressed immune system. The development of inflammatory processes causes significant changes in the metabolism of Cu^{2+} in humans and animals. These changes are generally considered as part of the defense mechanism evoked by the organism to bring the inflammatory reaction under physiologic control (Milanino et al., 1989). The rise in blood Cu^{2+} concentration is accompanied by a concomitant increase in ceruloplasmin concentration and these factors are highly correlated in animals experiencing inflammatory conditions (Conforti et al., 1982). Endotoxin, a potent stimulator of the acute phase response (APR), causes an increase in plasma ceruloplasmin. Calves administered LPS show an increase in concentration of plasma ceruloplasmin (Conner et al., 1989).

2.7.2. Immune responses to LPS.

Mucosal barriers. The mucosal membranes covering the respiratory, the digestive, the urogenital tracts as well as the eye conjunctiva, the inner ear and the ducts of all exocrine glands are equipped with powerful mechanical and chemical cleansing mechanisms that degrade and repel most foreign matter. In addition, a large and highly specialized innate and adaptive mucosal immune system protects these surfaces, and thereby the body interior, against potential insults from the environment. In a healthy animal, this local

immune system contributes almost 80% of all immune cells. These cells are accumulated in, or in transit between, various mucosa-associated lymphoid tissues (MALT), which together form the largest mammalian lymphoid organ system (Mestecky, 2005).

The mucosal immune system has three main functions: (i) to protect the mucous membranes against colonization and invasion by potentially dangerous microbes that may be encountered, (ii) to prevent uptake of undegraded antigens including foreign proteins derived from ingested food, airborne matter and commensal microorganisms, and (iii) to prevent the development of potentially harmful immune responses to these antigens if they do reach the body interior (Liebler-Tenorio and Pabst, 2006). At variance with the systemic immune apparatus which functions in a normally sterile milieu and often responds vigorously to invaders, the MALT guards the organs that are replete with foreign matter. Upon encountering of this variety of antigenic stimuli, the MALT selects appropriate effector mechanisms and regulates their intensity to avoid tissue damage and immunological exhaustion (Liebler-Tenorio and Pabst, 2006).

Mucosal immune responses. The MALT represents a highly compartmentalized immunological system and functions essentially independent from the systemic immune apparatus. It is comprised of anatomically defined lymphoid compartments such as the Peyer patches, the mesenteric lymph nodes, the appendix and solitary follicles in the intestine, and the tonsils and adenoids at the entrance of the respiratory and digestive tract, which serve as the principal mucosal inductive sites where immune responses are initiated (Mowat, 2003; Kiyono, 2004). Small but numerous clusters of immature

lymphocytes and dendritic cells have also been described in the subepithelial compartment of the mouse intestine and may represent sites of extrathymic lymphopoiesis (Ishikawa et al., 1999; Guy-Grand et al., 2003). The MALT also contains diffuse accumulations of large numbers of lymphoid cells in the parenchyma of mucosal organs and exocrine glands, which form the mucosal effector sites where immune responses are manifested. Consistent with a high degree of compartmentalization, the MALT is populated by phenotypically and functionally distinct B cell, T cell, and accessory cell subpopulations as compared with systemic lymphoid tissues, and has also developed strong restrictions upon lymphoid cell recirculation between mucosal sites (Cesta, 2006).

Antigens taken up by absorptive epithelial cells, and specialized epithelial cells (membrane, or 'M', cells) in mucosal inductive sites are shuttled to, or directly captured by, 'professional' antigen-presenting cells (APCs; including dendritic cells (DCs), B lymphocytes, and macrophages), and presented to conventional $CD4^+$ and $CD8^+$ T cells, all located in the inductive sites. Certain antigens may also be processed and presented directly by epithelial cells to neighboring intraepithelial T cells, including T cells with limited repertoire diversity (T cells and NKT cells). Immune responses in mucosal tissues are governed by the nature of the antigen, the type of APCs involved, and the local microenvironment. With most types of nonpathogenic antigens (*e.g.*, food proteins), the 'default' pathway for mucosal DCs and other APCs seems to be to generate T helper 2 (T_H2) and various regulatory T cell types of responses (Iwasaki and Kelsall, 1999); this usually also results in active suppression of systemic immunity—'oral tolerance.'

Antigens and adjuvants, including most pathogens, harboring motifs sensed by mucosal APCs as 'danger signals' (*e.g.*, Toll-like receptor (TLR) ligands), on the other hand, and proinflammatory conditions in general, favor the development of stronger and broader immune responses engaging both the humoral-secretory and cellular immunity effector arms and also do not lead to oral tolerance (Mowat, 2003, Bilsborough and Viney, 2004; Iwasaki and Kelsall, 1999). It has been widely assumed that the recognition of pathogens by TLRs on mucosal APCs was distinct from the response to the commensal flora, but recently it was found that microbial commensals are also recognized by TLRs under normal conditions, and that this interaction seems crucial for maintaining epithelial homeostasis in the gut (Rakoff-Nahoum et al., 2004).

The sensitized mucosal immunocytes, both B and T cells, leave the site of initial encounter with antigen (*e.g.*, a Peyer patch), transit through the lymph, enter the circulation and then seed selected mucosal sites, mainly the mucosa of origin, where they differentiate into memory or effector cells. The anatomic affinity of such cells seems to be largely determined by site-specific integrins ('homing receptors') on their surface and complementary mucosal tissue specific receptors ('addressins') on vascular endothelial cells (Kunkel and Butcher, 2003). In addition, chemokines produced in the local microenvironment promote chemotaxis toward mucosal tissues and regulate integrin expression on mucosal immunocytes, thereby controlling cell migration (Campbell et al., 2003). Of particular interest are recent studies indicating that mucosal DCs, in addition to presenting antigen to cognate T cells, can also influence their homing properties. Thus, mouse DCs isolated from mesenteric lymph nodes and Peyer patches, but not from spleen

and peripheral lymph nodes, increase the expression of the mucosal homing receptor 47 (Stagg et al., 2002; Mora et al., 2003) and CCR9 (Mora et al., 2003), the receptor for the gut-associated chemokine TECK/CCL25 on memory T cells, and license effector/memory $CD8^+$ T cells to home preferentially to the intestinal epithelium. Notably, DC imprinting of gut homing specificity has recently been shown to involve retinoic acid, which is uniquely produced by intestinal DCs, but not by DCs from other lymphoid organs (Iwata et al., 2004). Taken together, these observations may explain the notion of a 'common mucosal immune system' whereby immunocytes activated at one site disseminate immunity to remote mucosal tissues rather than to systemic sites. At the same time, because chemokines, integrins and cytokines are differentially expressed among mucosal tissues, this fact may also partly explain why, within the mucosal immune system, there is a significant degree of compartmentalization linking specific mucosal inductive sites with particular effector sites (*e.g.*, the gut with the mammary glands and the nose with the respiratory and genital mucosa).

Effector mechanisms. In addition to the barrier function, mechanical cleansing mechanisms, and different chemical antimicrobial factors or defensins provided by the lining epithelium of different mucosal tissues, the mucosa contains a number of other cells of the innate immune system, including phagocytic neutrophils and macrophages, DCs, NK cells, and mast cells. Through a variety of mechanisms these cells contribute significantly to host defense against pathogens (Yuan and Walker, 2004) and for initiating adaptive mucosal immune responses.

The adaptive humoral immune defense at mucosal surfaces is to a large extent mediated by secretory IgA (sIgA) antibodies, the predominant immunoglobulin class in human external secretions. The resistance of sIgA to proteases makes these antibodies uniquely suited for functioning in mucosal secretions. The induction of IgA against mucosal pathogens and soluble protein antigens is dependent on T helper cells (Lycke et al., 1987; Hornquist et al., 1995), although IgA immunity to commensal flora may be thymus independent (Macpherson et al., 2000) and of low affinity (Stoel et al., 2005). In humans, transforming growth factor (TGF)- β and interleukin (IL)-10 in concert with IL-4 have been shown to promote B-cell switch to IgA and differentiation into IgA-producing cells (Goodrich et al., 1998; Asano et al., 2004). In this regard, in addition to mucosal T cells, which produce large amounts of IL-4, IL-10 and TGF-β, human muco-epithelial cells provide a major source of TGF- β and IL-10, suggesting that cooperation between neighboring lymphocytes and epithelial cells in the mucosal microenvironment is pivotal for programming preferential maturation of IgA-committed B cells.

Although sIgA is the predominant humoral defense mechanism at mucosal surfaces, locally produced IgM and IgG, and in the lower respiratory tract and in the genitourinary mucosa, serum-derived IgG can also contribute significantly to immune defense. Mucosal cytotoxic T lymphocyte (CTL) responses have been described after oral, nasal, rectal or vaginal immunization (Klavinskis et al., 1996; Staats et al., 2001), and recently also after transcutaneous immunization (Belyakov et al., 2004). Mucosal CTLs have been shown to be crucial for the immune clearance of pathogens in several animal models of infection with enteric or respiratory viruses and intracellular parasites (Franco et al.,

1995; Bender et al., 1992; Buzoni-Gatel et al., 1997). In most studies, wild-type or attenuated viruses and bacteria have been required to induce CTLs in mucosal tissues. There are, however, exceptions to this rule, inasmuch as use of certain adjuvants such as cholera toxin and related enterotoxins can promote mucosal CTL development when administered orally or nasally with soluble proteins and peptides (Bowen et al., 1994; Simmons et al., 2001). Besides CTLs, interferon (IFN)-γ producing $CD4^+$ T cells, induced either by the live pathogens or by mucosal immunization with inactivated vaccines in combination with cholera toxin or other mucosal adjuvants, have been found to be important for mucosal immune defense to both viral and bacterial infections; their protective mechanism(s), however, remain to be defined (Johansson et al., 1997; Ermak et al., 1998; Harandi et al., 2001). Thus, appropriate adjuvants or delivery systems, or both, may critically favor the induction of protective mucosal cellular responses, and this notion is of importance for developing mucosal vaccines against intracellular pathogens or their toxins.

2.7.3. Mechanisms of lipopolysaccharide recognition and clearance.

Unlike many other bacterial toxins, Gram-negative bacterial endotoxin known also as LPS has no enzymatic or other intrinsic activity of its own. The 'toxicity' of LPS is conferred entirely by the injurious response of the animal to presence of endotoxin. When animals sense endotoxin, however, the usual outcome is a successful antimicrobial defense, not lethal toxicity (Munford, 2005. In adapting to ubiquitous presence of Gram-negative bacteria, animals evolved mechanisms that allow both rapid recognition of

endotoxin, as one way to sense invading bacteria and mobilize host defenses, and endotoxin detoxification to avoid tissue injury (Munford, 2005).

There is little doubt that endotoxin recognition plays an important role in antimicrobial host defense. Numerous studies indicate that animals that have developed mutations in MD-2 or Toll-like receptor 4 (TLR4), which form the LPS signaling receptor complex, are susceptible to Gram-negative bacterial infections (Cross et al., 1989). Research conducted in the last decade has established the significance of the body's endotoxin-detoxifying mechanisms.

Innate immune system. Inflammation plays an important role during infection and tissue damage. Inflammatory responses are initiated by innate immunity and do not require participation of the adaptive division of the immune system. The main goal of the innate immune response is to stop infection and eliminate invasive bacteria from the host and prevent development of clinical signs of inflammation (Heumann and Roger, 2002). However, in certain cases, the over response of the innate immune system may contribute to aggravation of pathological processes resulting in multiple organ failure, shock, and death (Heumann and Roger, 2002).

Inflammation involves recruitment of humoral and cellular components of the immune system. Presence of bacteria initiates humoral responses and activates immune cells, leading to production of a variety of mediators, cytokines, and chemokines. All these mediators initiate a series of events, including increased permeability of blood vessels,

exudation of fluids, and migration of leukocytes to the inflammatory site, which results in killing and clearance of invading pathogens (Heumann and Roger, 2002).

The innate immune system plays an important role in detection and recognition of pathogenic bacteria. While bacteria have developed sophisticated systems to adhere and colonize the host, the latter has developed mechanisms to detect pattern recognition systems in bacteria and kill them. Pattern-recognition systems are the basic structures used by innate immunity to discriminate between self and foreign intruders (Heumann and Roger, 2002).

Role of LPS in inflammatory responses. One of the main components of Gram-negative bacterial membrane is LPS. When Gram-negative bacteria grow or die, they release LPS in both free form and in complex with bacterial surface proteins. There is increasing evidence that LPS plays a critical role in the inflammatory process (Rietschel et al., 1994). The concept that LPS is the major pathogenic factor of Gram-negatives is based on studies demonstrating that LPS or lipid A is able to reproduce in experimental animals a symptomatology very similar to those induced by pathogenic bacteria.

Recognition of LPS by host immune cells. Once bacterial components are released, they activate host immune cells such as macrophages, neutrophils, and endothelial cells to generate an inflammatory response (Heumann and Roger, 2002). This response might be local or involve systemic immune responses. Recognition of bacterial components by host cells involves a specific mechanism. Under high concentrations of microbial compounds they interact directly with immune cells because of their glycolipidic nature;

however, under low physiological concentrations, they bind to and activate specific receptors on responsive cells (Heumann and Roger, 2002).

The first step in LPS processing and neutralization is dissociation of LPS aggregates by the LPS-binding protein (LBP) to form LPS/LBP complexes. The latter are transferred to another protein known as membrane CD14 (mCD14) present on monocytic cells, leading to their activation (Pugin et al., 1993c). The LPS also might be transferred to another blood protein, the soluble CD14 (sCD14) (Pugin et al., 1993c). This process represents the earliest step in monocyte activation by LPS.

The role of LPS-binding protein in LPS removal and neutralization. Because of its amphiphilic nature, the LPS molecules released from Gram-negative bacteria are present in aggregates. Spontaneous release of monomeric molecules of LPS and its binding to CD14 occurs at a very low rate (Hailman et al., 1994). However, de-aggregation of LPS molecules is initiated by plasma LBP. The LBP is an enzyme catalyzing movement of LPS monomers to mCD14 or sCD14 and high-density lipoprotein (HDL) (Hailman et al., 1994; Yu and Wright, 1996; Wurfel, et al., 1994; Tobias et al., 1993). Binding of LPS to CD14 activates immune cells whereas binding to HDL neutralizes LPS (Tobias, et al., 1999; Ulevitch and Tobias, 1994; Ulevitch and Tobias, 1999). Therefore, the host response to LPS depends on the rate of binding and internalization of LPS as well as on the rate of its neutralization by HDL. Interestingly, recent investigations indicate that LPS/LBP complexes bind to CD14 before LPS is transferred to HDL (Yu and Wright, 1996). This suggests that LPS first activates immune cells before it is neutralized by HDL to prevent overreaction of the immune system.

Role of mCD14 in LPS neutralization and removal. mCD14 is a glycoprotein located on the surface of the myelomonocytic cells. The mCD14 performs two main functions: (i) serves as a receptor for LPS; (ii) helps internalization of LPS molecules. These two functions of mCD14 have been revealed by observations that certain antibodies against mCD14 suppress activation of cells by LPS but do not interfere with its internalization whereas other antibodies suppress the process of LPS internalization without affecting cell activation (Gegner et al., 1995). Furthermore, these observations suggest that while LPS signal transduction and LPS clearance utilizes both LBP and mCD14, these two pathways diverge after LPS binding to mCD14. LPS is internalized within minutes into monocytes (Gallay et al., 1993), and the rate and degree of internalization increase with the size of LPS aggregates (Kitchens and Munford, 1998).

The role of mCD14 in activation of monocytic cells by LPS was established using antibodies to block CD14 function (Wright et al., 1990). In addition, transfection of CD14-negative cells with CD14 increased sensitivity to LPS (Lee et al., 1992). Studies in Cd14 gene knockout mice showed that they do not respond to low doses of LPS (Haziot et al., 1996). Thus, under physiological conditions, activation of monocytes by LPS involves creation of a ternary complex with LBP and mCD14 on the surface of monocytes (Gegner et al., 1995)

Role of soluble CD14 in LPS neutralization and removal. Besides the membrane-bound form of CD14, there is a second form of CD14, the soluble CD14. The latter is either shed from the membrane form of CD14 or is produced as free form of sCD14. Binding of sCD14 to LPS is a direct process that is catalyzed by LBP (Hailman et al.,

1994). The sCD14/LPS complex also may bind to cells that lack CD14 such as endothelial cells or some epithelial cells inducing production of cytokines (Tapping and Tobias, 1997; Pugin et al., 1993a). In fact, many epithelial cells do not respond to LPS even in the presence of sCD14 (Hedlund et al., 1996). Moreover, the role of sCD14-activation of endothelial and epithelial cells in pathophysiology of LPS remains to be defined in the future. In fact, exposing endothelial cells to LPS induces their activation by an indirect pathway involving cytokine production by CD14-positive monocytic cells (Pugin et al., 1993b).

Besides its role in LPS-induced activation of cells that lack CD14, the soluble form of CD14 can also antagonize LPS/LBP-induced activation of cells containing mCD14, by competing with mCD14 for binding of LPS. However, this process requires far higher concentrations of sCD14 than those present in blood (Haziot et al., 1994). This finding questions the inhibitory role of sCD14 on the response of immune cells containing mCD14 under physiological conditions. Another function of sCD14 is to facilitate the transfer of LPS to HDL, resulting in neutralization of LPS. This suggests that LBP and sCD14 function as molecules that shuttle LPS, having both activatory and suppressive effects via mCD14 and HDL, respectively.

Role of other molecules in removal and neutralization of LPS. There are several other molecules, besides CD14, on the surface of the monocyte/macrophage cells that recognize LPS. Among these molecules the scavenger receptor and the integrin CD11/CD18 receptor mediate uptake of LPS that leads to its detoxification. In fact, this process does not contribute to LPS-induced cell activation. CD18 might play a role in

activation of immune cells only at high concentrations of LPS, as indicated by observations in CHO cells transfected with CD11c/CD18 (Ingalls and Golenbock, 1995). However, there are no reports to indicate involvement of the scavenger receptor in LPS-induced cell activation.

Role of Toll-like receptors in LPS signal transduction. Immune cells have certain receptors known as pattern-recognition receptors that recognize conserved structures on microbial pathogens such as LPS. These structures play an important role in discriminating between self and nonself. This is one of the basic principles that the innate immunity operates, a concept suggested by Hoffmann and his colleagues (Hoffmann et al., 1999). Toll-like receptors (TLRs) and CD14 are good examples of pattern-recognition receptors. Interestingly, both TLRs and CD14 share repeated leucine-rich motifs in their extracellular portions, similar to other pattern-recognition proteins of the innate immune system. However, while TLRs are proteins embedded in the membrane through a transmembrane domain, CD14 lacks such a domain. Moreover, all TLRs have an extracellular domain rich in amino acid leucine as well as an intracellular conserved domain, the "Toll domain". So far, about ten different TLRs have been identified.

Involvement of TLR4/MD-2 in LPS signaling. The exact mechanism of how LPS induces signal transduction after binding of the LPS/LBP complex to CD14 has remained hypothetical for many years. Several lines of evidence suggested participation of CD14 in the early phase of the process. However, evidence indicated that CD14 was not involved in cell signaling since the GPI anchor of CD14 prevents signal transduction. It was hypothesized that another transmembrane protein might act in concert with CD14 in this role (Triantafilou and Triantafilou, 2002).

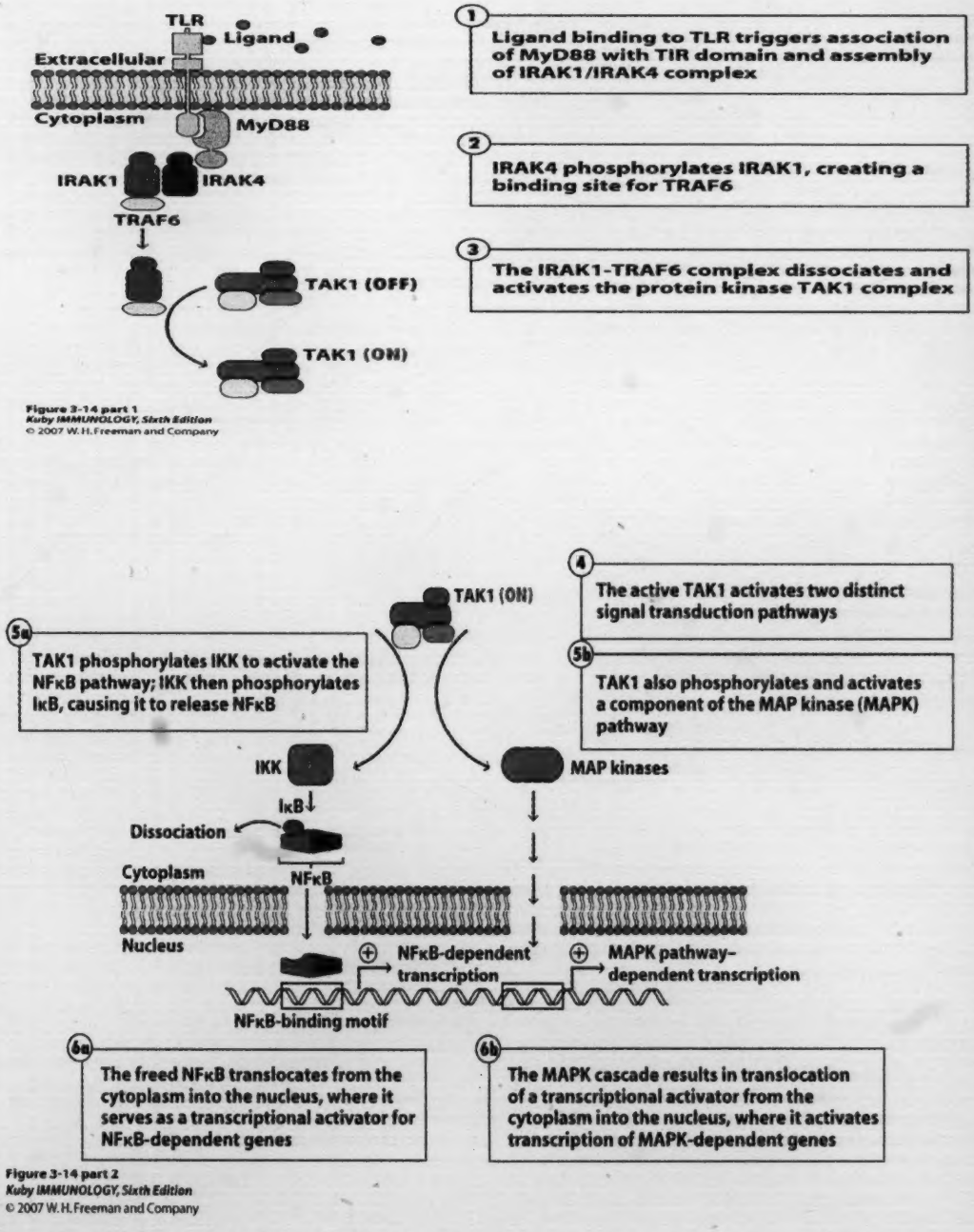
The postulated transmembrane protein transmitting the LPS signal inside the cell has now been identified as a member of the TLR family. The most likely candidates to transmit the LPS signal from membrane-bound CD14 to the cytoplasm are the recently identified TLRs, and in particular TLR4 (Anderson, 2000; Beutler, 2000).

Our understanding of LPS signaling relates to discoveries of several molecules involved in innate immunity in *Drosophila*, which is very resistant to microbial infections. Resistance of *Drosophila* to infections was related to the synthesis of potent antimicrobial peptides such as Toll molecules. The evidence for the role of TLR4 in LPS signaling came from the observation showing that C3H/HeJ mice resistant to LPS have a point mutation in the Toll domain of the *Tlr4* gene and that another LPS-resistant mouse strain, C57BL/10ScCr, exhibits a total deletion of the *Tlr4* gene (Poltorak et al., 1999). Interestingly, mice rendered deficient in the *Tlr4* gene (TLR4 knockout mice) do not respond to LPS (Takeuchi et al., 1999). Moreover, introduction of TLR4 in LPS unresponsive human embryonic kidney cells (HEK) restores LPS response (Chow et al., 1999). In addition, the response of HEK cells was amplified when they were cotransfected with MD-2, a molecule linking LPS and TLR4 (Shimazu et al., 1999).

MD-2 appears to play a significant role in LPS signaling. In fact, MD-2 is a secreted protein that lacks a transmembrane domain and forms heterogenous dimeric subunits (Visintin et al., 2001). Recent work has indicated that LPS binds directly to MD-2 without involvement of LBP and LPS is associated with at least three molecules TLR4, MD-2, and CD14 (da Silva Correia et al., 2001; Viriyakosol et al., 2001). Therefore, a

multimeric LPS receptor complex, involving at least CD14, TLR4, and MD-2 is necessary for LPS signaling.

Role of TLR4 in LPS signaling. Recent data indicate that there are two molecules that are sequentially recruited during TLR4 signaling (Figure 2.4), the adapter molecule MyD88 and the interleukin-1 (IL-1) receptor-associated kinase (IRAK). In fact, LPS signaling operates in a very similar way with IL-1 signaling. There are three molecules that bind together to form a complex during IL-1 signaling: the IL-1, IL-1 receptor I, and IL-1 receptor-associated accessory protein (IL-1R-AcP). On the other hand, the cytoplasmic MyD88 binds to this complex by Toll/Toll interactions. The IRAK molecule is then recruited to the complex. Then, IRAK is dissociated to interact with tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6). The latter interaction results in activation of the immune cells through nuclear factor-κB (NF-κB) or c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAP). These NF-κB and JNK/SAP pathways are involved in activation of various proinflammatory genes. It is well-known the important role that the MyD88 gene plays in these two pathways, since disruption of the MyD88 gene results in loss of IL-1 mediated function (Adachi et al., 1998) and in lack of response to LPS (Kawai et al., 1999).


TLR
Ligand
Extracellular
Cytoplasm
MyD88
IRAK1
IRAK4
TRAF6
TAK1 (OFF)
TAK1 (ON)
1 Ligand binding to TLR triggers association of MyD88 with TIR domain and assembly of IRAK1/IRAK4 complex
2 IRAK4 phosphorylates IRAK1, creating a binding site for TRAF6
3 The IRAK1-TRAF6 complex dissociates and activates the protein kinase TAK1 complex
Figure 3-14 part 1
Kuby IMMUNOLOGY, Sixth Edition
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TAK1 (ON)
4 The active TAK1 activates two distinct signal transduction pathways
5a TAK1 phosphorylates IKK to activate the NFκB pathway; IKK then phosphorylates IκB, causing it to release NFκB
5b TAK1 also phosphorylates and activates a component of the MAP kinase (MAPK) pathway
IKK
MAP kinases
IκB
Dissociation
Cytoplasm
NFκB
Nucleus
NFκB-dependent transcription
MAPK pathway–dependent transcription
NFκB-binding motif
6a The freed NFκB translocates from the cytoplasm into the nucleus, where it serves as a transcriptional activator for NFκB-dependent genes
6b The MAPK cascade results in translocation of a transcriptional activator from the cytoplasm into the nucleus, where it activates transcription of MAPK-dependent genes
Figure 3-14 part 2
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Figure 2.3. Ligands such as LPS activate TLR signaling pathway. Also, two molecules MyD88 and IRAK are required for signaling cascade. (Figure adopted from Kuby Immunology, 6th edition, 2007). IRAK - interleukin-1 (IL-1) receptor-associated kinase; TLR - Toll like receptor; TRAF- tumor necrosis factor (TNF) receptor-associated factor; TAK - TGF (Transforming growth factor) activated kinase; MyD88 - myeloid

differentiation primary response gene (88); IKK – Ikappa B kinases ; NF-κB- nuclear factor kappa B ; MAPK - Mitogen activated protein kinases.

2.8. Stimulation of mucosal immunity

2.8.1. Oral vaccination against pathogens or their toxins. Most pathogenic bacteria initiate infection by interacting with the host mucosa. After colonization of mucosal layers, some bacteria generate disease by releasing toxic compounds; others translocate into host tissues and give systemic or organ-specific diseases. Therefore, enhancing mucosal defenses is important for controlling bacterial infections or bacterial related diseases. Stimulation of local responses is possible through mucosal application of whole dead bacteria or their antigens because parenteral vaccination induces little or no IgA, the type of antibody that is secreted mostly in mucosal tissues and that is responsible for their defense (McGhee et al., 1992). In spite of advantages of mucosal application of vaccines, most of the vaccines in use today are parenteral vaccines and the few available mucosal ones are mainly based on application of whole microorganisms (Holmgren and Czerkinsky, 2005). To improve safety, today's vaccines are based mainly on bacterial-derived antigenic compounds.

Advantages of mucosal vaccination. Mucosal application of vaccines has several advantages over parenteral vaccination. First, mucosal application of the antigen stimulates production of IgA antibodies specific to that antigen at the site of infection. Second, because mucosal immunization elicits expression of mucosa-specific homing receptors for mucosal primed lymphocytes, the immunization at one mucosal site can induce specific responses at distant sites. This is important because protective immunity

could be induced in segregated mucosal sites in a practical way such as by oral or intranasal immunization. Third, mucosal vaccination affects systemic IgM and IgG, in addition to IgA responses, supplementing host defenses against invasion by bacteria or their products (De Magistris, 2006). The simultaneous induction of mucosal and systemic antibodies is advantageous especially in cases when pathogens infect the host through both systemic and mucosal route. In this case, a mucosal vaccine could be used to protect different categories of animals at risk of infection or intoxication. Moreover, mucosal vaccination could be utilized against pathogens that use both mucosal and non-mucosal routes of entry (i.e. blood or skin).

Besides stimulation of plasma IgG, IgM, and mucosal IgA antibodies, mucosal vaccination elicits cell-mediated immune responses involving $CD4^+$ T helper cells and $CD8^+$ cytotoxic T lymphocytes (De Magistris, 2006). The latter responses are important in combating intracellular pathogens. Therefore, mucosal immunizations have the potential to activate all the different arms of the immune system.

Mucosal administration of vaccines also confers a number of practical advantages. First of all mucosal administration is non-invasive and does not require the use of needles. This would increase vaccine compliance and would also avoid problems of blood transmissible infections due to needle re-use especially in developing countries (Levine, 2003). Moreover, mucosal vaccination is relatively easy and does not require expensive veterinary personnel. Reduced adverse effects and the potential for frequent boosting may also represent further advantages over injectable vaccines. Finally, production of mucosal

vaccines may be less expensive than that of injectable vaccines that require high standards of purity, in addition to sterility.

Mucosal immunization has been tested through many different mucosal sites; however, the most practical and promising routes for future vaccine development have proved to be the intranasal and the oral administrations (De Magistris, 2006). One should keep in mind that antigens such as proteins, polysaccharides, and DNA are degradable in the gastrointestinal tract and other mucosal layers and should be adequately administered and protected. The nasal mucosa is a very attractive site for application of mucosal vaccines because the environment is free of acids and degrading enzymes. Another advantage of nasal administration is the low amount of antigen/adjuvant needed to be applied to induce immune responses. Different devices have been developed over the years for nasal application of vaccines such as spray-based and bio-adhesive formulations in order to retain the vaccine in the nasal cavity. The oral route of immunization also has advantages if properly prepared and administered and if given in such amounts as to be retained in the oral cavity (De Magistris, 2006). If the oral formulation is such that the vaccine is swallowed into the stomach it raises problems of antigen dilution because of the large surface of the gastrointestinal tract and higher amount of antigen/adjuvant needed, with consequent high costs of production (Walker, 1994). Moreover, vaccines have to be targeted to specific sites of the epithelial cell surface to ensure their appropriate uptake. To these purposes several delivery systems have been developed. Most of them are particulate structures that may facilitate targeting of the antigen to M cells. Delivery systems can carry antigens and adjuvants and in some cases they act as adjuvants themselves.

Another advantage of mucosal immunization is that it is non-invasive and does not require the use of needles. This increases vaccine compliance and also avoids problems with blood transmission of infection especially in developing countries due to needle re-use (Levine, 2003). In addition, mucosal immunizations are associated with lower side effects when compared with vaccines delivered parenterally (Walker, 1994). Mucosal vaccines also could be delivered by farmers themselves.

2.8.2. Parenteral administration of LPS.

Induction of tolerance to LPS. The way an animal responds to endotoxin is a life or death phenomena. Indeed, animals that are not able to detect presence of endotoxin may die if they are infected by Gram-negative bacteria (Heumann and Roger, 2002). On the other hand, animals that are able to detect presence of endotoxin and respond too vigorously may also die from over reaction to endotoxin or bacterial presence (Heumann and Roger, 2002). The outcome of Gram-negative bacterial infection is thus determined not only by the ability to detect endotoxin and respond to its presence, but also by numerous individual responses that inactivate endotoxin and/or prevent harmful reactions to it. An interesting property of mammals is development of refractoriness to endotoxin known as 'endotoxin tolerance'. It is known that animals pretreated with a sublethal dose of LPS become tolerant to subsequent challenges with a lethal dose of LPS and display reduced mortality. Endotoxin tolerance is defined as a lowered capability of the host or of cultured immune cells (e.g., macrophages/monocytes) to respond to LPS following a previous exposure to this stimulus. Although early studies on endotoxin tolerance started over half a century ago (Beeson, 1946) to date our knowledge on the molecular

mechanisms of endotoxin tolerance has increased immensely. Since the discovery of the TLR4 protein as the major receptor for LPS, many investigators have focused on the role of TLR4 signaling pathway as a mechanism of endotoxin tolerance (Medzhitov, 1997). Changes of cell surface molecules, signaling proteins, pro-inflammatory and anti-inflammatory cytokines, and other mediator proteins related to endotoxin tolerance have been studied intensively (Ziegler-Heitbrock, 1995; West and Heagy, 2002).

During the years, LPS tolerance has been known by different names such as hyporesponsiveness, refractoriness, adaptation, de-activation, desensitization, immunoparalysis or reprogramming (Ziegler-Heitbrock, 1995; West and Heagy, 2002). One should keep in mind that LPS tolerance is not a total shut down of signaling pathways of immune cells. The LPS tolerant animals and cells can still respond to repeated LPS exposures and express specific genes and proteins (Learn et al., 2001). Therefore, the terminology that best describes the phenomena associated with repeated exposure to LPS is adaptation and reprogramming instead of tolerance.

In fact, the concept of endotoxin tolerance has been broadened recently and has been referred to as 'microbial tolerance' because other bacterial components are able to induce a similar state of low responsiveness. 'Microbial tolerance' is thought as a beneficial process because it limits the inflammatory response induced during an infection, and in so doing, protects the host from developing shock caused by excessive production of inflammatory cytokines by monocytes and macrophages. Indeed, the well-known state of

lack of endotoxic shock during Gram-negative sepsis is attributable to the development of such a refractory state (Ertel et al., 1995).

'Microbial tolerance' is manifested in the form of reduced capacity for antigen presentation, low levels of MHC class II expression, suppressed monocyte functions and cytokine production (Volk, 1991). Such a state is very suggestive of endotoxin-tolerant response and has, therefore, been proposed as a protective response to limit over activation of the immune system and to ease the shock syndrome.

Following administration of LPS the host responds with activation of immune cells and production of a variety of proinflammatory cytokines as well as with numerous metabolic changes such as fever, weight loss, shock, and lethality (Greismann and Woodward, 1965). Early studies have established two distinguished phases of LPS tolerance (Greismann et al., 1969). The first phase, known as 'early endotoxin tolerance', appears within hours after LPS treatment and lasts for up to a week. During this phase, there is a typical, non-specific, and antibody-independent suppression of macrophage functions to LPS from different strains of Gram-negative bacteria. The second phase of LPS tolerance is known as 'late phase endotoxin tolerance'. This refractory state develops weeks to months after LPS injection and is restricted to the LPS serotype applied initially, and is antibody-dependent, specific to O-antigen of LPS.

The early phase of LPS tolerance is considered to be of great importance in etiopathogenesis of sepsis. During this phase, macrophages play a crucial role in LPS

tolerance by decreasing secretion of macrophage-derived proinflammatory cytokines in animals made LPS-tolerant (Mengozzi and Ghezzi, 1993). Research also indicates that parenteral administration of LPS suppresses the ability of monocytes to respond to LPS and lowers secretion of pro-inflammatory cytokines and chemokines in response to LPS (Medvedev et al., 2000). During LPS tolerance there is suppression of cytokine and chemokine production and activation of MAPK, IκB kinases (IKK), transcription factors NF-κB and AP-1 as well as of IRAK-1 (Kratz et al., 1999; Kohler and Joly, 1997).

Although during LPS tolerance there is suppression of a variety of mediators in macrophages, tolerance is not a total anergy since there is unaffected or enhanced expression of several mediators. Lipopolysaccharide exerts most of its effects via the activity of macrophage mediators released in response to LPS stimulation. The inflammatory response is regulated by a complex network of mediators that directly interact with each other's expression or biological activity. In this context, a number of macrophage mediators such as IL-10, IL-1 receptor antagonist, tumor necrosis factor receptor (TNFR) II, TGFβ, and PGE_2 have potent anti-inflammatory activity by suppressing the formation of proinflammatory cytokines (Randow et al., 1995). Thus, it has been presumed that autocrine mechanisms are also involved in suppression of cytokine production during LPS tolerance.

LPS tolerance and protection from disease. Mounting evidence indicates that induction of 'endotoxin tolerance' provides protection against lethality and morbidity in animal models of endotoxemia and sepsis. Lipopolysaccharide tolerance is associated with

suppression of different cytokines and chemokines, a decrease in leukocyte infiltration, and consequently attenuation of disease. These findings suggest that induction of LPS tolerance might be useful in prevention of diseases related to endotoxin or bacterial infections in dairy cows postpartum (Cavaillon, 1995; Salkowski et al., 1998; Gustafson et al., 1995).

Mounting evidence indicates that 'endotoxin tolerance' might be beneficial in preventing harmful effects of Gram-negative bacteria. Rowley (1955) was the first to describe increased resistance of mice to bacterial infection after administration of *Escherichia coli* cell wall extracts 48h prior to challenge. The same protection was afforded when isolated endotoxin was injected instead of cell walls (Rowley, 1956). Subsequently, this phenomenon of reduced susceptibility after endotoxin application was extended to infections with other bacterial species and even some viral pathogens (Shilo, 1959). Pretreatment with endotoxin or cellular components of Gram-negative bacteria induced nonspecific protection against infection with a number of different extra- and intracellular bacteria including both Gram-negative and Gram-positive species (Shilo, 1959; Parant, 1980a, 1980b). More recently, increased resistance of LPS-pretreated animals to lethality and organ damage associated with multi-germ sepsis, induced e.g. by the heat-shock protein CLP was reported (Urbaschek et al., 1984; Urbaschek and Urbaschek, 1985; Neviere et al., 1999). Experiments performed by Rayhane et al. (2000) corroborated the notion that increased resistance is nonspecific by demonstrating improved survival and decreased fungal burden of LPS pretreated mice with disseminated *Cryptococcus neoformans* infection.

On the other hand, concern has been raised whether suppression of inflammatory responses during LPS tolerance would interfere with normal host defense and thus predispose animals to infectious diseases (Cavaillon, 1995). Indeed, host defense against infection with small numbers of replicating pathogens requires an intact cytokine response to halt proliferation and dissemination of the pathogen (Nakano et al., 1992; Mastroeni et al., 1999). In contrast to models of acute hyperinflammation such as endotoxic shock, neutralization of proinflammatory cytokines worsens the outcome of infection with low numbers of virulent bacteria (Tite et al., 1999; Dai et al., 1997) and many cytokine-deficient mice that are resistant to inflammatory damage rapidly succumb to otherwise sublethal infections (Mastroeni et al., 1999; Pfeffer et al., 1993; Rothe et al., 1993). Also, mice inherently hyporesponsive to LPS because of a nonfunctional mutation in the TLR4 gene (Qureshi et al., 1999; Poltorak et al., 1998) display increased susceptibility to Gram-negative pathogens (Cross et al., 1995; Hagberg et al., 1984). Furthermore, experimentally induced endotoxin tolerance displays features of immunoparalysis observed frequently in post-septic or post–traumatic patients several days or weeks after systemic inflammation: Monocytes from immunoparalysed patients were impaired in their ability to produce TNF-alpha upon restimulation with LPS *in vitro* (Wilson et al., 1997) and displayed diminished surface expression of MHC II (Hershman et al., 1999). These cellular defects were associated with an increased incidence of infectious complications and lethal outcome of disease (Hershman et al., 1999). Since similar alterations of monocyte/macrophage activity were found during experimentally

induced LPS tolerance, it was feasible that induction of LPS tolerance equally interfered with host defense.

2.9. Endotoxin-related diseases and exploration of novel preventing strategies: A synthesis

Dairy cows are affected by one or multiple metabolic diseases immediately after calving including acidosis, laminitis, displacement of abomasum, fatty liver, ketosis, milk fever, downer cow syndrome, retained placenta, infertility, bloat, liver abscesses, mastitis, unexplained fever, and metritis (Drackley, 2005). Dairy farmers in Alberta lose approximately $132/cow per year for treatment of metabolic and infection diseases in addition to milk loss in the first 30 days of lactation (Heikkila and Van Biert, 2007). Twenty four percent of all dairy cows in Canada, registered with Dairy Herd Improvement (DHI), are culled for a number of reasons; however, 79% of those cows are culled for metabolic related conditions (i.e.: 30% for infertility, 17% for mastitis, 11% for feet and leg problems, 10% for low milk production, 9% for unspecified sickness, 1% for LDA, and 1% for milk fever; Source: CanWest DHI & Velacta, 2007).

Our knowledge on the etiology and pathogenesis of metabolic diseases has grown tremendously; however, our comprehension of the causative agent(s) is far from complete. The fact that metabolic diseases are still present in almost half of the cows in a dairy herd is a clear indication that there is a lack of proper understanding of causative agent(s) and this has hampered our efforts to develop a successful strategy for prevention of metabolic disorders. Understanding the role of endotoxin in development of metabolic

diseases and exploring novel ways to prevent its negative effects would be very beneficial to dairy industry to avoid high veterinary bills and great losses in milk production and culled cows.

The most interesting observation about metabolic diseases is that they are interrelated (Erb et al., 1985); however the reason for their association is not correctly understood. Different investigators have suggested that endotoxin released in the rumen fluid or other mucosal layers might be the common causative agent of metabolic disturbances in transition dairy cows (Nagaraja et al., 1978; Boosman et al., 1991; Aiumlamai et al., 1992; Kindhal et al., 1992; Dohmen et al., 2000; Fuerll et al. 2002; Andersen et al., 2003; Mateus et al., 2003; Kaze et al., 2004; Ametaj et al., 2005; Gozho et al., 2007, Emmanuel et al., 2008).

Dr. Ametaj's team at University of Alberta is intensively working to identify the role of endotoxin in development of metabolic diseases and results conducted recently indicate that feeding high-grain diets and the release of large amount of endotoxin in the rumen fluid might be the initial stimulus that starts the cascade of metabolic perturbations in dairy cows (Emmanuel et al., 2008). Moreover, recent research by Dr. Ametaj and his collaborators demonstrated, in a Ussing chamber system, that acidic pH similar with that of rumen acidosis might facilitate translocation of endotoxin through rumen and colon mucosal layers into the host's circulation (Emmanuel et al., 2008). Developing novel ways to prevent translocation of endotoxin into the host circulation would be very

beneficial to prevent development of multiple metabolic disturbances in postpartal dairy cows.

Therefore the objectives of the following studies were to determine whether 1) repeated oral exposure of transition dairy cows during 2 wk before and 1 wk after parturition would be able to prevent metabolic disturbances in postpartal dairy cows as well as improve their overall health status; 2) repeated intravenous administration of endotoxin during 2 wk before and 1 wk after parturition would induce tolerance to endotoxin and therefore prevent development of metabolic perturbations or aggravate health status of dairy cows.

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Chapter 3

Oral Administration of Lipopolysaccharide from *Escherichia coli* 0111:B4 Prevents Metabolic Disturbances in Periparturient Dairy Cows

Abstract

Endotoxin released in the ruminal fluid of transition dairy cows fed high proportions of grain has been implicated in multiple metabolic disorders like laminitis, fatty liver, liver abscesses, and displaced abomasum. Prevention of endotoxin translocation into the blood circulation might avoid harmful effects of endotoxin. It is known that repeated exposure of mucosal epithelium to bacterial antigens is associated with induction of tolerance to that antigen. Therefore, the objective of this research was to study the effects of repeated oral administration of lipopolysaccharide (LPS) on immune response, plasma metabolites, health status and productivity in transition dairy cows. Sixteen pregnant multiparous Holstein dairy cows were assigned to 2 treatment groups 2 weeks before the expected day of parturition. Cows were administered orally, twice per week, for 3 consecutive weeks starting 2 weeks before parturition the following treatments: 3 mL of saline (control), or 3 mL of saline containing LPS from *E. coli* 0111:B4. The amount of LPS administered each week was 0.01, 0.05, or 0.1 μg/kg BW (~650 kg cow). Blood samples were obtained from the jugular vein twice per wk for 3 consecutive weeks (2 weeks before and 1 week after parturition) minutes before administration of the treatment as well as once per week during week 2, 3, and 4 after parturition and plasma serum amyloid A (SAA), lipopolysaccharide binding protein (LBP), haptoglobin, anti-LPS immunoglobulin A (IgA), immunoglobulin G (IgG) and immunoglobulin M (IgM) as

well as cortisol, non-esterified fatty acids (NEFA), beta-hydroxy butyric acid (BHBA), glucose, lactate, insulin cholesterol, calcium (Ca^{2+}), iron (Fe^{2+}), zinc (Zn^{2+}), and copper (Cu^{2+}) were measured. Records of all clinical metabolic diseases were obtained beginning 2 wk before and up to 8 wk after parturition. Clinical data showed lower incidence of metabolic disorders in cows treated with oral LPS. No differences in acute phase proteins (SAA, LBP and haptoglobin) were obtained. Plasma IgA was not different between the two groups whereas IgG was lower and IgM was greater in cows treated with oral LPS. Plasma cortisol also did not differ between the two treatment groups. On the other hand, concentrations of NEFA and BHBA in plasma increased linearly in control cows during transition period reaching peak values 2-5 wk postpartum, respectively; however, both NEFA and BHBA changed only slightly and remained within normal ranges in cows treated with oral LPS. Furthermore, plasma glucose, lactate, and insulin were greater in vaccinated cows. No effect of treatment was obtained for plasma cholesterol. Plasma Ca^{2+} and Fe^{2+} did not differ between the control and treated cows; however, plasma Zn^{2+} increased whereas plasma Cu^{2+} decreased in cows treated with oral LPS. In addition, no differences were obtained in DMI between the two groups; however, LPS-treated cows produced more milk than the control group. Taken together results of this study indicate that repeated oral administration of LPS from *Escherichia coli* 0111:B4 during peripartum improved immune status and prevented clinical and metabolite disturbances typically observed in dairy cows around parturition. This suggests a causal role for endotoxin in the etiology and pathogenesis of metabolic disorders in dairy cows and the potential utilization of oral treatment against LPS for prevention of endotoxin translocation and its harmful effects in periparturient dairy cows.

3.1. Introduction

Metabolic disorders are a group of diseases that affect one in two dairy cows within the first month after parturition (Drackley et al., 2005). The most frequent metabolic diseases of dairy cows are the following: 1) subacute- and acute- ruminal acidosis, 2) laminitis, 3) ketosis, 4) fatty liver, 5) left displaced abomasum (LDA), 6) milk fever, 7) metritis, 8) mastitis, 9) retained placenta, 10) bloat, 11) downer cow syndrome, 12) milk fat depression syndrome, 13) udder edema, 14) liver abscesses, and 15) sudden death syndrome (Erb et al., 1985; Erb and Gröhn, 1988; Gröhn et al., 1989). Interestingly, most metabolic disorders are interconnected with each other. For example, Erb et al. (1985) showed that cows affected by milk fever are more prone to mastitis, retained placenta, metritis, LDA, and ketosis. Moreover, cows affected by acidosis are more prone to laminitis, displaced abomasum, milk fever, mastitis, and fatty liver (Goff, 2006). In addition, cows affected by retained placenta are more prone to metritis, LDA, and ketosis (Erb et al., 1988). Also, ketosis and fatty liver are common findings in cows affected by milk fever, mastitis, laminitis, displaced abomasum, metritis, retained placenta, and udder edema (Gröhn et al., 1989; van Dorp et al; 1999). Although there is a speculation that there might be a common etiological factor behind this association the exact cause for this relationship is not understood well at present.

Feeding high grain diets might be the initial stimulus that disturbs metabolic processes appearing as metabolic disorders (Ametaj et al., 2005). In fact, the high levels of milk production, during the last decades, were achieved mainly by increasing the proportion of

grain content in cow's diet. Feeding high proportions of grain has been demonstrated to cause several major changes in dairy cows such as: 1) a steady decline in ruminal pH to acidotic levels, 2) a drastic change in the microbial ecology of the gastrointestinal tract, and 3) the release of large amounts of endotoxin in the ruminal fluid. Emmanuel et al. (2008) showed that feeding dairy cows increasing proportions of grain at 0, 15, 30, and 45% of the ration dry matter (DM) was associated with lower ruminal pH as the amount of grain in the diet increased. Moreover, Russell et al. (2001) demonstrated major changes in the composition of ruminal and gastrointestinal microflora in favor of Gram-negatives during feeding of diets rich in grain. Furthermore, Andersen et al. (1994) and Emmanuel et al. (2008) demonstrated 14- to 18-fold increase in the amount of endotoxin in the rumen fluid of cows fed high amounts of grain.

Although rumen acidosis and endotoxin have been implicated in the etiology of multiple metabolic disorders, the mechanism(s) involved was not clear until recently. In a very elegant experiment, Emmanuel et al. (2007) demonstrated that under acidic conditions and in presence of LPS from *Escherichia coli* B: 055 the permeability of rumen and colon tissues to ^{3}H-mannitol increased 4-5 fold. This was the first demonstration that rumen acidosis was able to increase rumen and colon permeability to non-nutritious compounds and, thus, makes the gastrointestinal tract 'leaky' (Emmanuel et al., 2008). Interestingly, Emmanuel et al. (2008) also reported that translocation of endotoxin through rumen and colon tissues were independent of pH. Endotoxin was able to penetrate the mucosa when present in the solution. This suggested that endotoxin released during feeding of high-grain diets might translocate through mucosal walls.

Besides the gastrointestinal route, endotoxin might gain access to blood circulation in a number of other conditions. For example, cows with naturally occurring acute coliform mastitis develop bacteremia, and, thus, indirectly introduce endotoxin into the blood circulation (Wenz et al., 2001). Endotoxin also was detected in the plasma of cows with endometritis (Mateus et al., 2003). Feeding high-grain diets resulted in microlesions of ruminal papillae in sheep (Kay et al., 1969; McManus et al., 1977), which may also contribute and facilitate translocation of endotoxin into the blood stream. In addition, presence of endotoxin in the blood was demonstrated in cattle and sheep after induced grain engorgement (Dougherty et al., 1975; Andersen et al., 1994). Translocation of endotoxin is associated with multiple immunologic, endocrine, and metabolic perturbations as well as deterioration of health status of the host (Culbertson and Osburn, 1980).

We hypothesized that if we induce mucosal immunity against LPS by immunizing periparturient dairy cows orally with LPS we might be able to avoid translocation of endotoxin into the blood stream and prevent metabolic disturbances related to endotoxin. Therefore, the objective of this investigation was to study the effects of repeated oral immunization with increasing doses of LPS from *Escherichia coli* 0111:B4, two weeks before and one week after calving, on health status as well as immunologic, metabolic, mineral, and production responses of periparturient dairy cows.

3.2. Materials and methods

Animals and treatments. Sixteen clinically healthy and nearing term heifers and multiparous Holstein cows were equally distributed to two different groups of 8 animals each. There was slight difference in the age and parity while the randomization was done. Each group was assigned one treatment either oral lipopolysaccharide (LPS) or oral saline (controls). Administration of LPS was done twice per week during 2 weeks before and 1 week after parturition in weekly increasing doses of 0.01 μg, 0.05 μg, and 0.1 μg/kg BW on day 1 and 3 of each week. The lowest dose of 0.01 μg/kg BW of LPS was chosen because previous experiments have shown minimal clinical changes in dairy cows at this dose. The highest dose of 0.1 μg/kg BW of LPS was selected because the maximum host response was observed at this dose in the previous experiments. The initial crystalline *Escherichia coli* LPS (Lipopolysaccharide-FITC from *E.coli* strain 0111:B4 supplied by Sigma-Aldrich Canada Ltd.) containing 10 mg of purified LPS was dissolved in 10 mL of distilled water as suggested by the manufacturer and stored in the refrigerator. The dose of LPS was dissolved in 3 mL of saline and was introduced into the oral cavity of the cows using disposable syringes. The same amount (i.e., 3 mL) of carrier (saline) was orally sprayed to all cows in the control group. Experimental procedures were approved by the University of Alberta Animal Policy and Welfare Committee and animals were cared for in accordance with the guidelines of the Canadian Council on Animal Care (1993).

For each sampling period, all cows were moved to maternity barn and were restrained to take blood samples from an intravenous catheter introduced an hour before sampling into

the jugular vein. Ten mL of blood was collected through a plastic syringe and poured gently into a tube containing Na-EDTA (Preanalytical Systems Beliver Industrial Estate, Plymouth, UK). Blood samples were put immediately in ice, centrifuged within 20 min (Rotanta 460R, Hettich Zentrifugan, Tuttlingen, Germany), and plasma was separated and stored at -20°C until analyses. Immediately after blood withdrawal indwelling catheters were filled with 2 to 3 mL of 0.85% sterile saline containing 50 IU of heparin (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) to prevent clot formation. Blood was withdrawn twice per week beginning week -2 and -1 before the expected day of parturition and at 1 week after parturition as well as once per week beginning week 2, 3, and 4 after parturition. No stress responses from cows were observed during blood withdrawal.

Monitoring of animals for clinical parameters. Rectal temperature, respiratory rate, and rumen contractions were measured before treatment as well as every hour, up to 6 hours after administration of treatment. Feed intake was recorded daily during the entire experimental period. All disease and medication history was recorded for each cow throughout the entire experimental period.

3.3. Sample analysis

3.3.1. Plasma acute phase proteins.

Serum amyloid A. Concentration of SAA in plasma was determined by commercially available ELISA kit (Tridelta Development Ltd., Greystones C., Wicklow, Ireland) with monoclonal antibodies specific for SAA coated on the walls of the microtitre strips

provided. Samples were initially diluted 1:500 and if some of the samples had optical density values below the range of the standard curve they were reanalyzed in lower dilutions. All samples were tested in duplicate and the optical density values were read on microplate spectrophotometer (Spectramax 190, Molecular devices Corporation, CA) at 450 nm. The detection limit of the assay was 18.8 ng/mL.

Lipopolysaccharide-binding protein. A commercially available ELISA kit was used to quantify plasma LBP (Cell Sciences, Inc., Norwood, MA, USA). The antibody used to coat the wells cross-reacts with bovine LBP. Plasma samples were initially diluted 1:500 and samples with optical density values lower than the range of the standard curve were tested with a lower dilution. Samples were tested in duplicate and the optical density at 450 nm was measured on a microplate spectrophotometer (Spectramax 190, Molecular devices Corporation, CA, USA).

Haptoglobin. Concentrations of haptoglobin in the plasma were measured with an ELISA kit provided by Tridelta Development Ltd. (Greystones C., Wicklow, Ireland). The detection limit of the assay was 0.25 ng/mL as defined by the linear range of the standard curves. All samples were tested in duplicate and the optical density of 630 nm was used to measure on a microplate spectrophotometer (Spectramax 190, Molecular devices Corporation, CA, USA).

3.3.2. Plasma immunoglobulins.

Immunoglobulin A, IgG, and IgM. The immunoglobulins IgA, IgG, and IgM were measured using commercially available ELISA kit EndoCab which has been developed for determination of endotoxin core antibodies. The EndoCab ELISA is solid-phase enzyme-linked immunosorbent assay based on the sandwich principle with a working time of 2 ½ h.The color developed is proportional to the amount of anti endotoxin core antibodies present in the sample. The absorbance is measured at 450 nm with a spectrophotometer. The minimum detection levels of IgG, IgM, and IgA EndoCab antibodies are 0.0125 GMU/mL, 0.055 MMU/mL, and 0.156 AMU/mL.

3.3.3. Plasma metabolites

Cortisol. Plasma cortisol was measured by commercially available EIA kit (Diagnostic Systems Laboratories, Inc, Webster, Texas, USA). The procedure involved the basic principle of enzyme immunoassay where there is a competition between an unlabeled antigen and an enzyme-labeled antigen for a fixed number of antibody binding sites. The amount of enzyme-labeled antigen is inversely proportional to the concentration of the unlabeled analyte present in the solution. Unbound materials were removed by decanting and washing the wells. All samples were in duplicate. The optical density was measured at 450 nm in a microplate reader (Spectramax 190, Molecular devices Corporation, CA, USA) and the concentration was calculated by using a four parameter curve fit.

Non-esterified fatty acids. Quantitative determination of plasma NEFA was done by an enzymatic colorimetric method using kits provided by Wako Chemicals (Richmond, VA,

USA). The principle of the test involves acylation of coenzyme A by fatty acids in the sample in presence of acyl-CoA synthetase and production of hydrogen peroxide in presence of acyl-CoA oxidase. Hydrogen peroxide, in presence of peroxidase, permits the oxidative condensation of 3-methyl-N-ethyl-N-β-hydroxy ethyl-O-aniline (MEHA) with 4-aminoantipyrine to form a purple colored adduct which is proportional to the NEFA in the sample. The test was modified as described by Johnson and Peters (1993). Duplicates were used to read plasma contents at an optical density of 550 nm, on a microplate spectrophotometer (Spectramax 190, Molecular devices Corporation, CA, USA). The lower detection limit of the assay was 0.50 mEq/L.

Beta-hydroxy butyric acid. Enzymatic measurement of BHBA by β-hydroxybutyrate dehydrogenase was used for measuring plasma concentration of BHBA using a commercially available kit (Stanbio Laboratory, Boerne, TX, USA). The principle of the test involves conversion of BHBA in the samples to acetoacetate and NADH at pH 8.5 by β-hydroxybutyrate dehydrogenase in the presence of NAD. The NADH produced reacts with INT in the presence of diaphorase to produce a color proportional to the concentration of BHBA in the sample. Controls and blanks were used in this procedure to eliminate the difference in the color intensity. Plasma BHBA was measured in duplicates by reading on a microplate spectrophotometer (Spectramax 190, Molecular devices Corporation, CA, USA) at an optical density of 505 nm. The lower detection limit of the assay was 125 mmol/mL.

Glucose. Concentration of glucose in plasma was quantified by an enzymatic method by a kit provided by Diagnostic Chemicals Ltd. (Charlottetown, PE, USA). The procedure involved phosphorylation and oxidization of glucose in samples resulting in the production of NADH which produces a color proportional to the glucose concentration in the sample. All samples were tested in duplicate and plasma glucose was then determined by reading on a microplate spectrophotometer (Spectramax 190, Molecular devices Corporation, CA, USA) at an optical density of 340 nm. According to the manufacture's instructions the lower detection limit of the test was 0.06 mg/dL.

Lactate. Concentration of lactate in plasma was determined using commercially available lactate assay kits (Biomedical Research Service Center., Buffalo, NY, USA). The principle of the test involves reduction of tetrazolium salt INT in a NADH-coupled enzymatic reaction to formazan which exhibits a red color whose intensity is proportional to concentration of lactate. The procedure was modified as described by Johnson and Peters, 1993. The lactate standard provided in the kit was diluted to set a detection range of 125 to 1,000 μM. Reading of all samples was done in duplicate at an optical density of 492 nm and on a microplate spectrophotometer (Spectramax 190, Molecular devices Corporation, CA, USA).

Insulin. Insulin was measured in the plasma samples by commercially available ELISA kit supplied by Mercodia AB (Mercodia, Uppsala, Sweden). This procedure involved a solid phase two-site enzyme immunoassay. It is based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants

on the insulin molecule. During incubation, Insulin in the sample reacts with peroxidase-conjugated anti-insulin antibodies and anti-insulin antibodies bound to the micro titration wells. After simple washing that removes unbound enzyme labeled antibody, the bound conjugate is detected by reaction with 3, 3'-5, 5'-tetramethylbenzidine (TMB). The reaction is stopped by the addition of acid, giving colorimetric endpoint that can be read spectrophotometrically (Spectramax 190, Molecular devices Corporation, CA, USA) at 450 nm.

Cholesterol. Plasma cholesterol was measured using kits provided by Diagnostic Chemicals Ltd. (Charlottetown., PE, USA). The colorimetric method is based on the principle of hydrolyzing cholesterol esters to free cholesterol and oxidation of free cholesterol to cholest-4ene-3-one with simultaneous production of hydrogen peroxide. The hydrogen peroxide couples with 4-aminoantyrine and p-hydroxybenzoate, in the presence of peroxidase to yield a chromogen whose intensity is proportional to concentration of cholesterol in the sample. Samples were prepared in duplicates and the reading of plasma cholesterol was conducted at optical density of 505 nm on a microplate spectrophotometer (Spectramax 190, Molecular devices Corporation, CA, USA).

3.3.4. Plasma minerals.

Concentrations of Ca^{2+}, Fe^{2+}, Zn^{2+}, and Cu^{2+} in plasma were measured by atomic absorption spectrometry (Spectr AA800, Varian, Sidney, Australia). Plasma samples were diluted 100 times for estimation of Ca^{2+} and 10 times for Zn^{2+}, Fe^{2+}, and Cu^{2+}. For estimation of plasma Ca^{2+} acetylene with nitrous oxide burner was used and for plasma

Zn^{2+}, Fe^{2+}, and Cu^{2+} acetylene burner only was used. The upper detection limit of the instrument was at 5.0 mg/L for Ca^{2+}, Cu^{2+}, and Fe^{2+} and 2.0 mg/L for Zn^{2+}.

3.4. Statistical analysis

The MIXED procedure of SAS (SAS Institute Inc., Cary, NC, USA Version 9.1.3) was used to analyze the blood variables. The repeated measures design was used by using the following model:

$\mathbf{Y = \mu + T_i + W_j + TW_{ij} + e_{ijkl}}$ where **T** = treatment, **W** = week, **TW** = Interaction of treatment and week, $\mathbf{e_{ijkl}}$ = random error.

The PDIFF option was used to compare the LS means. Measurements of same animal were considered as repeated measures. The cow within **T x W** was used as the experimental unit. The covariance structure of the repeated measurements for each variable was modeled separately according to the lowest values of fit statistics based on the BIC (Bayesian information criteria). The significance of results was declared at $P < 0.05$.

3.5. Results

3.5.1. Acute phase proteins

Serum amyloid A. Concentration of SAA in plasma was not different between the two treatment groups ($P > 0.05$; Figure 3.1). However, there was an increase in SAA in both groups immediately after parturition ($P < 0.05$). Thus, plasma SAA increased almost 3 fold from the week before to the week after parturition in both groups (i.e., treatment

group from 12,738 ng/mL to 37,794 ng/mL and control group from 17,029 ng/mL to 51,320 ng/mL, respectively). Thereafter, concentrations of SAA decreased gradually until week six after parturition. Also, statistical analysis indicated no treatment by time interaction ($P > 0.05$).

Lipopolysaccharide binding protein. Statistical processing of the data indicated no differences in the concentration of LBP in plasma between the treated and the control group (Figure 3.2). Results also showed an effect of time on plasma LBP ($P < 0.05$). Similarly with plasma SAA, concentration of LBP increased in both groups immediately after parturition. Thus, concentration of LBP the week before and the week after parturition was 20,674 to 39,242 ng/mL for the oral LPS group and 19,056 to 65,876 ng/mL in the saline treated group. No treatment by week interaction was obtained for plasma LBP ($P > 0.05$).

Haptoglobin. No differences between the two treatment groups were obtained regarding concentration of haptoglobin in plasma (Figure 3.3). However, time had an effect on plasma haptoglobin ($P < 0.05$). Haptoglobin concentration increased from 141 µg/mL to 891 µg/mL and from 218.65 µg/mL to 788 µg/mL between the week before and after parturition in the treated and control group, respectively. Similarly to plasma SAA and LBP, concentration of haptoglobin declined after parturition reaching the lowest levels at 6 weeks after calving. No interaction between treatment and week was evidenced ($P > 0.05$)

3.5.2. Plasma immunoglobulins.

Immunoglobulin A. No treatment or time effect was obtained for plasma endotoxin IgA antibodies ($P > 0.05$ and $P > 0.05$; Figure 3.4). In fact, plasma levels of endotoxin IgA antibodies ranged in both groups from the lower level of 0.04 MU/mL to the upper concentration of 0.07 MU/mL during the whole experimental period. Also, no treatment by week interaction was evidenced for plasma IgA ($P > 0.05$).

Immunoglobulin G. Results indicated differences between plasma endotoxin IgG antibodies between the two treated groups with control group having greater concentrations from immediately after parturition up to six weeks after parturition ($P < 0.001$; Figure 3.5). There was also a time effect for plasma IgG with values increasing continuously from the lower concentrations before parturition to the greater concentration on week 6 after calving ($P < 0.05$). No treatment by week interaction was observed between the two treated groups ($P > 0.05$).

Immunoglobulin M. Plasma endotoxin IgM antibodies were greater in control cows versus cows treated with oral LPS during both the week before and after parturition ($P < 0.01$; Figure 3.6). No week effect or treatment by week interaction was obtained regarding plasma IgM ($P > 0.05$).

Cortisol. No differences between plasma concentrations of cortisol were detected between the two treated groups ($P > 0.05$; Figure 3.12) A week effect was obtained with regards to plasma cortisol with greater concentrations two weeks before calving and

declining almost 3 fold the week before calving and remaining at this levels for the remaining of the experiment ($P < 0.01$). No treatment interaction was obtained for plasma cortisol ($P > 0.05$).

3.5.3. Plasma metabolites

Non-esterified fatty acids. Concentration of NEFA in plasma was greater in the control cows starting one week before up to two weeks after parturition ($P < 0.01$; Figure 3.8). There was also a week effect for plasma NEFA with peak levels two weeks after parturition in both control animals and those treated with oral LPS at 1,153 mg/mL and 627 mg/mL, respectively ($P < 0.01$). No treatment by week interaction was obtained for plasma NEFA ($P > 0.05$).

Beta-hydroxy butyric acid. Plasma concentrations of BHBA was greater in saline-treated cows starting from the week before parturition ($P < 0.01$; Figure 3.9). Concentration of BHBA reached peak levels in control animals five weeks after parturition at 1,679 mg/mL whereas the highest concentration in cows treated with oral LPS was on week four at 683 mg/mL. No week effect or treatment by week interaction was obtained for plasma BHBA ($P > 0.05$).

Glucose. Concentration of glucose in plasma was greater in cows treated with oral LPS versus the control group ($P < 0.01$; Figure 3.7). No week effect or treatment by week interaction was evidenced for plasma glucose ($P > 0.05$ and $P < 0.05$).

Lactate. Lactate in plasma was greater in dairy cows treated with oral LPS than in control cows ($P < 0.05$; Figure 3.11). Plasma lactate reached a peak levels on week two after parturition in LPS treated cows with average values of 3,952 mg/mL and dropped to similar levels with control animals on week two after parturition and remained at those levels for the remainder of the experiment. There was no week effect or treatment by week interaction regarding plasma lactate ($P > 0.05$).

Insulin. Plasma insulin was greater in cows treated orally with LPS when compared to the control ones ($P < 0.01$; Figure 3.13). Insulin was higher two weeks before parturition and continuously decreased in control cows; however, no such declining trend was observed for cows treated orally with LPS. No week effect or treatment by week interaction was evidenced for plasma insulin ($P > 0.05$ and $P > 0.05$).

Cholesterol. Cholesterol in plasma was not different among the control and the treated cows ($P > 0.05$; Figure 3.10). However, there was a week effect obtained with plasma cholesterol with values starting to increase by the end of week two and continuously rising up to week 6 postpartum ($P < 0.01$), There was no treatment by week interaction regarding plasma cholesterol ($P > 0.05$).

3.5.4. Plasma minerals

Calcium and iron. There were no differences between plasma concentrations of Ca^{2+} and Fe^{2+} between the control and treated groups ($P > 0.05$ and $P > 0.05$; Figure 3.14 and Figure 3.15, respectively). There were week effects for both $Ca^{2+}$and Fe^{2+} between the

two treatment groups ($P < 0.05$ and $P < 0.05$, respectively). Plasma Ca^{2+} declined below 10 mg/mL immediately after parturition in both groups of cows and returned to normal afterwards. Plasma Fe^{2+} increased slightly the week before parturition and remained at those concentrations for the remaining of the experiment. No interaction between week and treatment were evidenced for plasma Ca^{2+} and Fe^{2+} ($P > 0.05$ and $P > 0.05$, respectively).

Zinc and copper. Both plasma Zn^{2+} and Cu^{2+} were affected by treatment administered to cows. However, while plasma Zn^{2+} was greater in cows treated with oral LPS plasma Cu^{2+} was lower in treated animals versus those of controls ($P < 0.001$ and $P < 0.01$; Figure 3.16 and Figure 3.17, respectively). There were no week effects or treatment by week interactions regarding plasma Zn^{2+} and Cu^{2+} ($P > 0.05$ and $P > 0.05$, respectively).

Rectal temperature. There was no treatment effect ($P > 0.05$) or week effect ($P > 0.05$) with respect to rectal temperature during the entire experimental period (Figure 3.18). In addition, there was no evidence of treatment by week interaction regarding this clinical variable ($P > 0.05$).

Dry matter intake. There was no treatment effect on dry matter intake between the group of cows treated with oral LPS and those treated with oral saline ($P > 0.05$; Figure 3.19). No week effect or treatment by week interaction was obtained for DMI ($P > 0.05$).

3.6. Discussion

Several metabolic diseases of dairy cows such as fatty liver, milk fever, laminitis, displaced abomasum, infertility, and mastitis have been linked to translocation of endotoxin into the blood stream (Hakogi et al., 1989; Boosman et al., 1991; Aiumlamai et al., 1992; Foley and Schlafer, 1994; Andersen, 2003; Ametaj et al., 2005; Emmanuel et al., 2008). However, to our best knowledge, there are no known strategies to prevent endotoxin-related diseases in dairy cows. The study reported herein was undertaken to evaluate whether repeated oral administration of increasing doses of LPS from *Escherichia coli* 0111:B4 in dairy cows around parturition would be able to prevent occurrence of metabolic diseases related to endotoxin and improve health status in periparturient dairy cows.

According to our hypothesis, repeated oral treatment with LPS prevented incidence of metabolic diseases in the treated cows during the first month after parturition (Table 3.1). Only one treated cow showed signs of lameness 36 days after calving and the same cow had mastitis at 56 days after calving. On the other hand, several cows in the control group were affected by one or multiple metabolic diseases as shown in Table 3.1. Moreover, one cow from the control group was culled because she became a downer cow.

Results of this study also showed that oral LPS did not affect concentration of plasma SAA, LBP, and haptoglobin. All three acute phase proteins increased in both groups of cows immediately after parturition. Plasma acute phase proteins are part of a general non-specific immune response and are produced by the liver under the stimulation of

proinflammatory cytokines such as IL-1, IL-6, and TNF-alpha (Kushner, 1993; Mackiewicz et al., 1991). Two of the proteins, SAA and LBP, are released into the systemic circulation or in mucosal layers to bind and neutralize endotoxin, whereas haptoglobin dampens the severity of cytokine release by macrophages and protects against endotoxin effects (Kushner, 1982; Arredouani et al., 2005). The reason for the peak plasma SAA, LBP, and haptoglobin in all cows on the first week after calving might be the proinflammatory cytokines coming from the Gram-negative contaminated tissues. For example, it is known that most cows develop uterine infections after calving (Hirvonen et al., 1999). Proinflammatory cytokines released in response to Gram-negative infections of the uterus are absorbed into the systemic circulation (Kucharski et al., 2008). Cytokines, then, induce production of acute phase proteins in hepatocytes (Mackiewicz et al., 1991). In fact, our data are in agreement with previous reports of enhanced acute phase proteins in dairy cows after calving (Jafari et al., 2006) and confirm previous findings that parturition is associated with mounting of an acute phase response (Humblet, et al., 2006; Emmanuel et al., 2008).

We did not measure endotoxin in plasma of our experimental cows. The reason for that is that endotoxin is quickly removed from systemic circulation by liver hepatocytes as well as liver and spleen macrophages, even if administered intravenously in large amounts (Mathison and Ulevitch, 1979; Shao et al., 2007). Research indicates that clearance of endotoxin from systemic circulation is affected by the health status of the host. Indeed, Andersen et al. (1996) reported measurement of plasma endotoxin only in the cows that spontaneously developed hepatic lipidosis but not in the healthy ones. Because of the

aforementioned difficulties, different investigators in human and rodent research measure plasma cytokines as indirect indicators of plasma endotoxin (Dofferhoff et al., 1992). Unfortunately, there are no bovine cytokine kits available. Therefore, measurement of anti-LPS immunoglobulins was used in our experiment as an indirect indicator for presence of endotoxin in the plasma of cows.

Results showed that plasma anti-LPS IgA antibodies were numerically lower in treated cows; however, the difference did not reach significance. This was expected because oral immunization usually stimulates production of secretory IgA in the mucosal membranes but not plasma IgA. Similar results were reported in mice treated orally with LPS to prevent sepsis induced by cecal ligation and puncture (Márquez-Velasco et al., 2007). Generally, IgA is found in all external secretions, like bile and intestinal fluids, tears, saliva, milk or other mucosal membranes (Woof and Kerr, 2006). Oral administration of antigen has been shown to induce preferentially antigen-specific IgA responses in mucosa-associated tissues. On the other hand, subcutaneous or intramuscular immunization results in IgM and IgG responses in systemic lymphoid tissues (Jeurissen et al., 1985; Mestecky and McGhee, 1989).

Indeed plasma anti-LPS IgG and IgM antibodies were affected by oral administration of LPS. Interestingly, results indicated that plasma IgG antibodies were lower in cows treated with oral LPS. This is an important indication that oral exposure to LPS developed mucosal immunity and prevented translocation of LPS into the host blood circulation. These findings may relate to the phenomenon of oral tolerance, in which oral

immunization with an antigen results in the development of suppressor T cells which inhibit IgG responses. Indeed, Richman et al. (1981) showed that orally fed antigen (e.g., ovalbumin) induced IgG-specific suppressor T cells that lowered systemic IgG. The contrary was true for the control cows in our experiment, which had greater concentration of anti-LPS IgG antibodies in their plasma. This indicates that control cows developed a secondary humoral immune response characterized by development of memory B cells to LPS. It is speculated that this might be related to translocation of endotoxin from gastrointestinal tract into the blood circulation of control cows.

Another interesting finding of this research was that plasma anti-LPS IgM antibodies were greater in the LPS-treated cows. Greater concentrations of plasma anti-LPS IgM antibodies suggest that oral exposure to LPS stimulated the primary humoral immune response in the treated group. These data are in agreement with previous research indicating that pretreatment of mice with oral LPS increased plasma anti-LPS IgM and protected them from sepsis induced by cecal ligation and puncture (Márquez-Velasco et al., 2007). The protective role of IgM in sepsis models has been reported to be critical for clearance of circulating LPS (Boes et al., 1998). Immunoglobulin M is important not only as a primary response to LPS but also for mucosal immunity. This type of immunoglobulin has been shown to play a significant role in mucosal immunity together with secretory IgA (Brandzaeg, 1996). Our findings demonstrated that oral LPS was able to modulate systemic humoral immune responses in transition dairy cows.

Different investigators have demonstrated that activation of immune system by intravenous administration of endotoxin in ruminants is associated with stimulation of the hypothalamo-pituitary-adrenal axis and the release of cortisol in systemic circulation (Coleman et al., 1993; Battaglia et al., 1998). Based on the latter findings we included measurement of plasma cortisol in our study to evaluate whether oral exposure to LPS would induce the release of cortisol into the blood stream. Data from our study showed that concentration of cortisol in the plasma was numerically greater in cows treated with oral LPS; however, the difference did not reach significance. Thus, the results suggest that repeated oral administration of LPS was not associated with entrance of LPS into the systemic circulation of the experimental cows and, therefore, was not able to induce the activity of the hypothalamo-pituitary-adrenal axis.

A high-producing dairy cow normally loses weight during lactation as energy output exceeds energy intake (Coppock, 1985). In fact, energy reserves must be utilized for production of milk constituents, especially milk fat (Grummer, 1995). Lipolysis occurs in the animal's adipose tissue and creates elevated concentrations of free fatty acids (i.e., NEFA) in the plasma (Brockman, 1979). An increased supply of NEFA to the liver favors increased production of BHBA and acetoacetate or ketone bodies (Brockman, 1979). High concentrations of NEFA and BHBA in the plasma are common findings in periparturient cows and usually are associated with enhanced incidence of fatty liver and ketosis, respectively (Baird, 1982; Drackley, 1999; Goff 2006; Guo et al., 2007). Interestingly, data from this research showed that both plasma NEFA and BHBA were lower in cows treated orally with LPS during the whole experimental period. Other

research involving intravenous administration of LPS in heifers or cows indicated that endotoxin modulates plasma NEFA and BHBA. Thus, Steiger et al. (1999) and Waldron et al. (2003) reported that intravenous LPS increased NEFA in the plasma quadratically and lowered plasma BHBA. Our data, in fact, do not fit with both the reported enhanced NEFA and BHBA in transition cows and with experiments involving intravenous administration of LPS. Our data support a new hypothesis that endotoxin might play a role in development of both ketosis and fatty liver in dairy cows and that oral treatment with LPS could prevent translocation of endotoxin and development of both diseases. Indeed, the immediate metabolic response of the liver to inflammation is a shift towards catabolism. Initially, glycogen is metabolized to glucose to sustain glycemia in response to decreased food intake, and fat oxidation is increased to provide energy and alternative fuels (ketone bodies). Following glycogen depletion, glycemia is maintained by gluconeogenesis from lactate and amino acids.

During early lactation plasma concentrations of insulin are lower than at any other time during bovine lactation (Hayirli, 2006). Furthermore, the lowest concentrations of insulin in plasma seen during early lactation occur in cows with the lowest blood concentrations of glucose (Herbein, 1985; Vazquez-Anon et al., 1994). Not only are insulin concentrations low, but insulin responsiveness also is impaired (Sano et al., 1993). An important finding of this investigation was that plasma concentration of three metabolites representing energy metabolism such as insulin, glucose, and lactate were greater in cows treated with oral LPS compared to those treated with oral saline. This suggests that LPS-treated cows were in a better energy balance than control cows. In fact, insulin is an

anabolic hormone and acts to preserve nutrients in their storage forms by stimulating glycogenesis, lipogenesis, and glycerol synthesis and by inhibiting gluconeogenesis, glycogenolysis, and lipolysis (Brockman and Laarveld, 1986). Insulin is also a potent regulator of feed intake and nutrient partitioning in ruminants (Laarveld et al., 1981).

It is not clear how oral administration of LPS improved energy status in postpartal cows; however, it is possible that this might be related to prevention of endotoxin translocation into blood. In fact, injection of endotoxin in animal models is associated initially with an increase in plasma glucose and then with a chronic state of hypoglycemia (Lang et al., 1985). Following acute administration of endotoxin, glycogenolysis is the primary source of glucose (Meinz et al., 1998); however, during chronic endotoxemia if glycogen stores are depleted, an increase in gluconeogenesis contributes to glucose production (Lang et al., 1994, McGuinness, 1994). On the other hand, it is known that prior exposure to endotoxin decreases the capacity of the liver to synthesize glucose (Ceppi et al., 1992, Filkins and Cornell, 1974, Morikawa et al., 1998,). Again, data of this research support our hypothesis that endotoxin translocation into blood stream plays a significant role in multiple dysfunctions during transition period and oral treatment of dairy cows with LPS has the potential to prevent negative energy balance observed in dairy cows around parturition.

Plasma cholesterol was not different between the two treatment groups. However, plasma cholesterol increased steadily in both group after calving. These data are in agreement with previous research indicating enhanced plasma cholesterol in dairy cows after

parturition (Ametaj et al., 2005). Cholesterol is an important precursor to many steroid hormones and bile acids. Enhanced cholesterol after calving supports cow's needs for precursors of reproductive hormones as well as for bile acids important in digestion of lipids and neutralization of endotoxin in the digestive tract.

Intravenous administration of LPS is associated with decreased plasma Ca^{2+} and Fe^{2+} (Waldron et al., 2003; Jacobsen et al., 2005). Calcium and Fe play important roles in the toxic effects of endotoxin, and both Ca antagonists and Fe chelators have been shown to improve survival in animals challenged with endotoxin (Hotchkiss et al., 1995; Kontoghiorghes and Weinberg, 1995). Results of this study showed that plasma concentrations of Ca^{2+} and Fe^{2+} did not differ between the two experimental groups. However, plasma Ca^{2+} declined in both groups immediately after parturition confirming previous reports of Ca^{2+} drop in postpartal cows (Goff, 2006). We are not sure whether the decline in plasma Ca is a protective measure of the host to bacterial infections postpartum or if it is a response to acidosis initiated by high-grain diets. Feeding diets with high proportions of rapidly fermentable carbohydrates causes rumen acidosis (Stacy and Wilson, 1970; Scott and Buchan, 1981). Rumen acidosis and acidemia are associated with elimination of large amounts of Ca^{2+} in the urine as an effective means of neutralizing and eliminating acid compounds from the organism (Jackson and Hemken, 1994).

The periparturient period is a collection of physiological events that combine to have a profound effect on the immune system. Periparturient immunosuppression is manifested

in a wide range of immunological dysfunctions, including impaired neutrophil and lymphocyte functions (Kehrli et al., 1989; Shuster et al., 1996; Mehrzad et al., 2001). As a result translocation of Gram-negative bacteria and infections of different tissues such as uterus and mammary gland occur (Sheldon et al., 2006). Pathogenic microbes require Fe^{2+} for growth and proliferation. Upon infection, microbes produce proteins, called sidephores, designed to strip serum divalent metals away from host proteins. Higher vertebrates respond to infection by increasing the expression of proteins that sequester serum iron away from bacteria. As a result, a diversion of Fe^{2+} traffic from the circulation to storage sites of the reticuloendothelial system occurs and host plasma Fe^{2+} levels decrease especially during the initial phases of infection or inflammation. Iron withholding can be an important mechanism for suppressing potentially pathogenic microbial growth and proliferation. Results from several studies have shown that infection can be intensified by injecting iron and thus negating host iron withholding defense mechanisms (Weinberg, 1978). Mice injected with LPS or turpentine, to induce an inflammatory state, showed an upregulation of the gene encoding for Fe^{2+} regulatory peptide hepcidin (Niocolas et al., 2002). Similarly, human hepatocyte cultures express hepcidin after exposure to LPS or medium from LPS activated macrophages. The proinflammatory cytokine IL-6 induces expression of hepcidin as well as the condition of hypoferremia. Among the many cytokines released from macrophages exposed to LPS, IL-6 is the main mediator of hepcidin upregulation during inflammation (Van Amersfoort et al., 2003). Infections with Gram-negative or Gram-positive bacteria are associated with a decline in plasma Fe^{2+} as bacteria require Fe^{2+} for their growth and to exert their pathological effects (Bullen, 1981; Lauffer, 1992). Results of this study did not show

differences between the two treatment groups. However, the group of cows treated with LPS had a 30% decline in plasma Fe^{2+} postpartum whereas the control group did not show peripartal changes in plasma Fe^{2+}. It is not clear whether this decline was in response to administration of increasing doses of LPS or whether LPS exposure conferred protection against bacterial infections to cows by decreasing plasma Fe^{2+}. Further research is warranted to evaluate the role of Fe^{2+} decrease after calving in protection of cows from bacterial infections.

Results of this study showed that plasma Zn^{2+} was greater in cows treated with oral LPS. Zinc is an important trace mineral essential for normal functioning of many enzymes in the organism. For example, Zn^{2+} is a component of enzymes that function as dehydrogenases, proteases, and esterases; functioning as an aid to maintaining the conformation of the aforementioned enzymes (Williams, 1989). Usually, plasma Zn^{2+} is withdrawn from blood during inflammatory conditions and mounting of an acute phase response (Kushner, 1982; Shenkin, 1995). Pro-inflammatory cytokines released during acute phase response have a direct influence on Zn^{2+} homeostasis. It has been shown that IL-6 induces the expression of the Zn^{2+} transpress porter Zrt- and Irt-like protein (ZIP) 14, thereby increasing zinc uptake into hepatocytes (Liuzzi et al., 2005). Interleukin-6 also upregulates the zinc binding protein metallothionein (MT) and increases cellular Zn^{2+} in hepatocytes (Schroeder and Cousins, 1990). Experiments with MT knockout mice confirmed that during endotoxin-induced inflammation, MT is required for Zn^{2+} sequestration in the liver, leading to a significant reduction in plasma Zn^{2+} levels (Philcox et al., 1995). Hence, during the acute-phase response, hypozincemia is caused by ZIP-

mediated translocation of Zn^{2+} into the liver and sequestration bound to MT. Conversely, Zn^{2+} affects several aspects of monocyte signal transduction and the secretion of pro-inflammatory cytokines by these cells (Haase and Rink, 2007), and Zn^{2+} supplementation has been shown to reduce the production of TNF-alpha and IL-1 in healthy humans (Prasad et al., 2004). Therefore, lower plasma Zn^{2+} in control cows suggests that there was translocation of endotoxin in those cows. In contrast, greater plasma Zn^{2+} in treated cows is indicative of prevention of endotoxin translocation in the LPS group.

In contrast to Zn^{2+} behavior, plasma Cu^{2+} decreased in cows treated with oral LPS. Generally, plasma Cu^{2+} increases during inflammatory conditions (Kushner, 1982; Shenkin, 1995). This trace element is a component of multiple anti-oxidant enzymes such as ceruloplasmin, catalase, and superoxide dismutase indispensable to fight side effects of endotoxin (Watson et al., 1999). The antioxidant enzymes also are important in defending the body against free radicals as well as toxic substances by converting them to a form that can be readily excreted. Lower plasma Cu^{2+} in the LPS-treated cows suggests that no translocation of LPS occurred in that group.

Clinical observations demonstrated that both groups of cows did not have differences in rectal temperature, respiratory rate (data not shown), and rumen contractions per minute (data not shown) during the entire experimental period. Our data are in agreement with previous research conducted by Cort et al. (1990) which showed no effects of intragastric LPS on rectal temperature or respiratory rate in male goats. Based on clinical effects of

oral LPS it is concluded that administration of LPS from *Escherichia coli* 0111:B4 is safe to be used orally in periparturient dairy cows.

Data regarding DMI showed no differences between the two treatment groups. These data are in line with other research conducted in ruminant animals indicating that intragastric LPS (20 mg/animal) had no effect on their feed intake (Cort et al., 1990). On the other hand, research involving intravenous administration of 2 μg/kg of BW in heifers demonstrated that LPS lowered feed intake (Steiger et al., 1999). The lack of effect of oral LPS on DMI suggests that oral application of LPS during the peripartum is safe to be used in transition dairy cows.

In conclusion, repeated oral administration of LPS from *Escherichia coli* 0111:B4 prevented incidence of clinical metabolic diseases. Cows treated orally with LPS developed a humoral immune response characterized by lower anti-LPS IgG and greater anti-LPS IgM antibodies. Also, the LPS-treated cows had lower NEFA and BHBA as well as greater glucose, lactate, and insulin in plasma indicating that oral LPS improved energy metabolism in the treated cows. In addition, the LPS-treated cows had better mineral status with greater Zn^{2+} and lower Cu^{2+} in plasma. Overall, our data support a role for endotoxin in the etiology and pathogenesis of multiple diseases in periparturient dairy cows and suggest utilization of repeated oral administration of LPS during the transition period to prevent endotoxin-related diseases in dairy cows.

3.7. Literature cited

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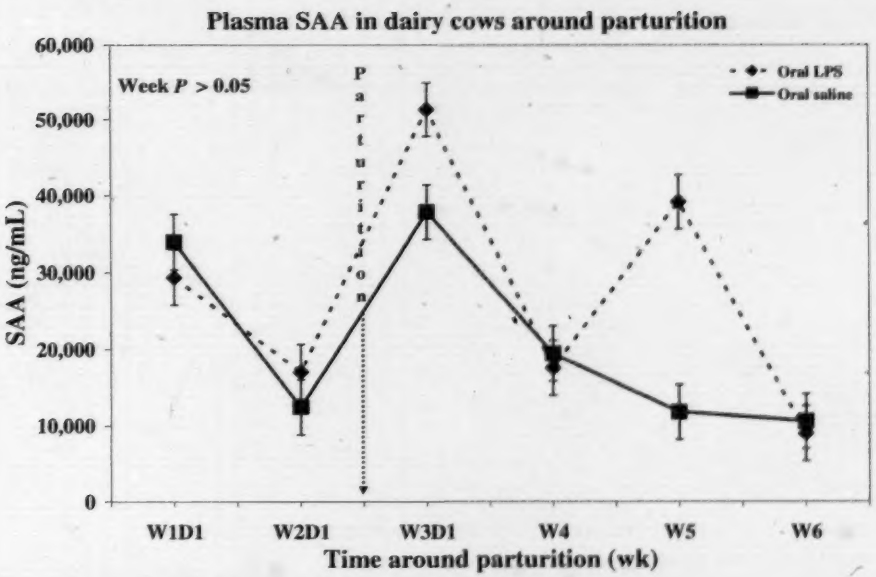

Figure 3.1. Concentration of SAA in the plasma of periparturient dairy cows treated orally with saline (control) or lipopolysaccharide (LPS) twice per week during -2 and -1 week before (W1 and W2) as well as 1 week after calving (W3). Blood samples also were collected once per week on week 2, 3, and 4 after calving (W4, W5, and W6).

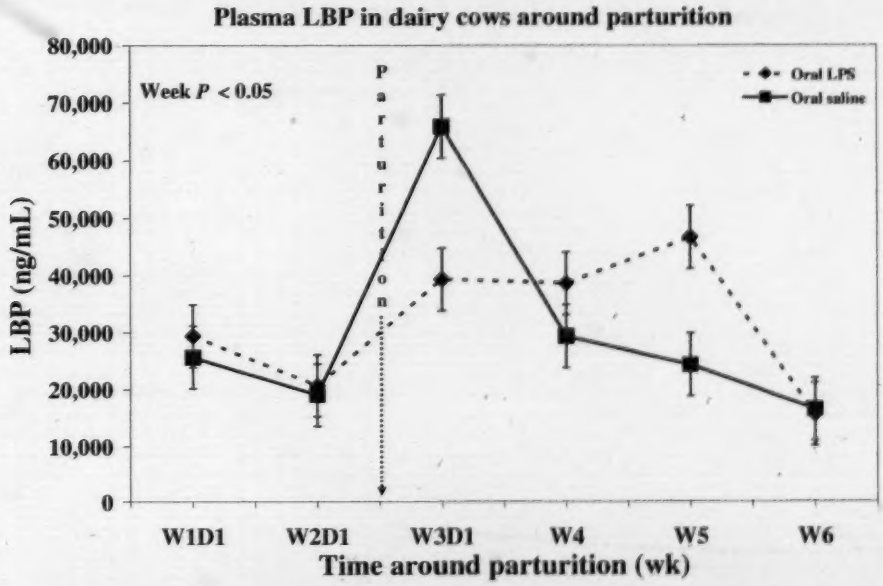

Figure 3.2. Concentration of LBP in the plasma of periparturient dairy cows treated orally with saline (control) or lipopolysaccharide (LPS) twice per week during -2 and -1 week before (W1 and W2) as well as 1 week after calving (W3). Blood samples also were collected once per week on week 2, 3, and 4 after calving (W4, W5, and W6).

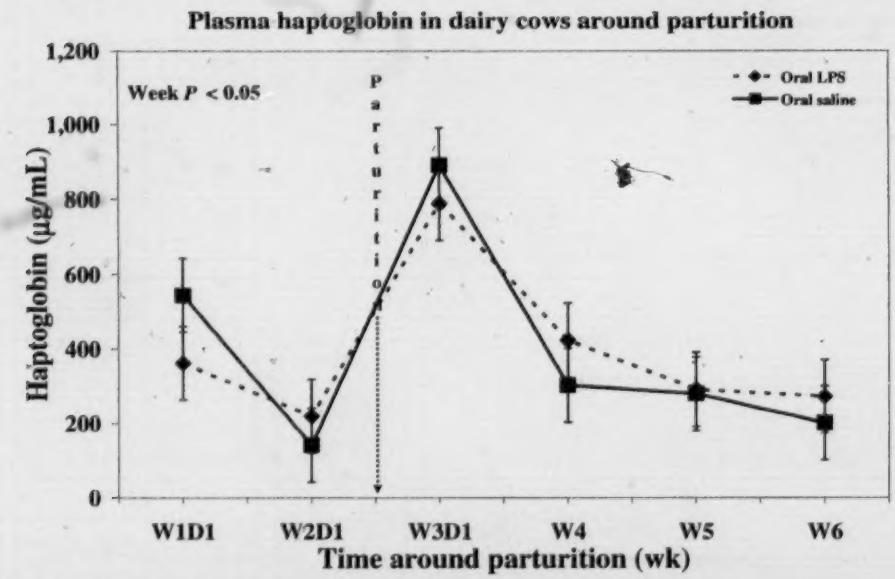

Figure 3.3. Concentration of haptoglobin in the plasma of periparturient dairy cows treated orally with saline (control) or lipopolysaccharide (LPS) twice per week during -2 and -1 week before (W1 and W2) as well as 1 week after calving (W3). Blood samples also were collected once per week on week 2, 3, and 4 after calving (W4, W5, and W6).

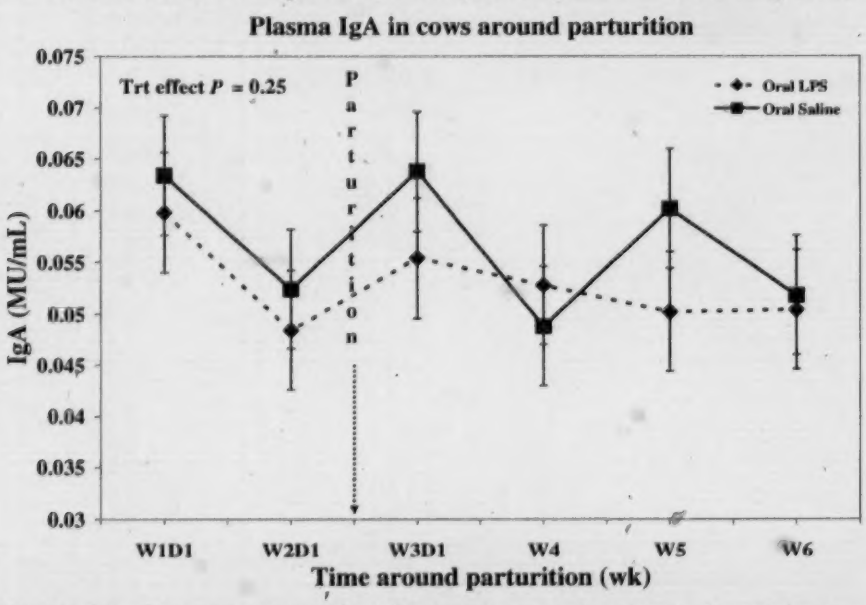

Figure 3.4. Concentration of IgA in the plasma of periparturient dairy cows treated orally with saline (control) or lipopolysaccharide (LPS) twice per week during -2 and -1 week before (W1 and W2) as well as 1 week after calving (W3). Blood samples also were collected once per week on week 2, 3, and 4 after calving (W4, W5, and W6).

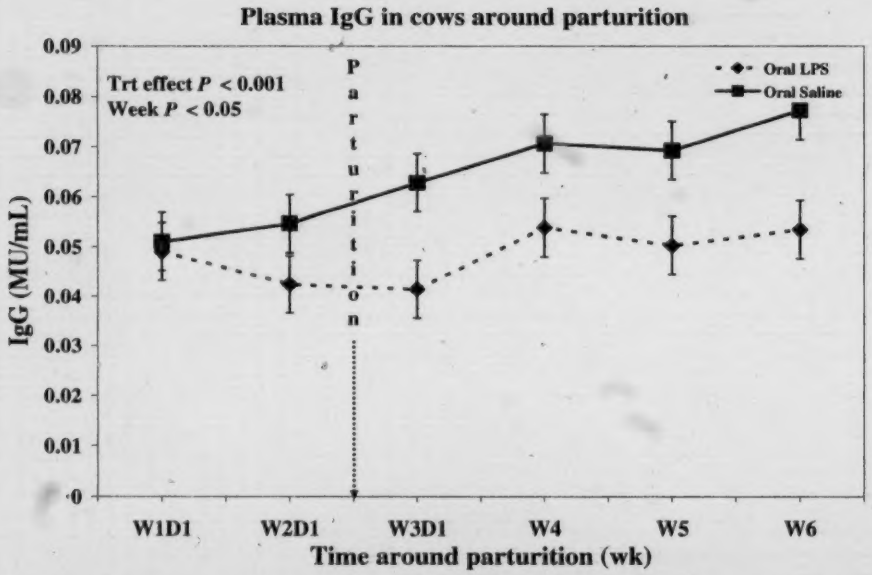

Figure 3.5. Concentration of immunoglobulin G in the plasma of periparturient dairy cows treated orally with saline (control) or lipopolysaccharide (LPS) twice per week during -2 and -1 week before (W1 and W2) as well as 1 week after calving (W3). Blood samples also were collected once per week on week 2, 3, and 4 after calving (W4, W5, and W6).

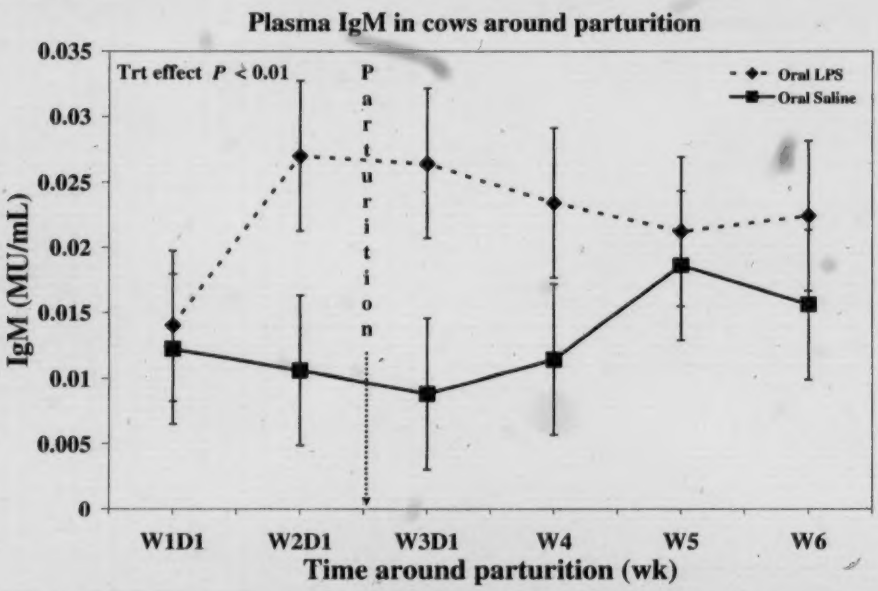

Figure 3.6. Concentration of IgM in the plasma of periparturient dairy cows treated orally with saline (control) or lipopolysaccharide (LPS) twice per week during -2 and -1 week before (W1 and W2) as well as 1 week after calving (W3). Blood samples also were collected once per week on week 2, 3, and 4 after calving (W4, W5, and W6).

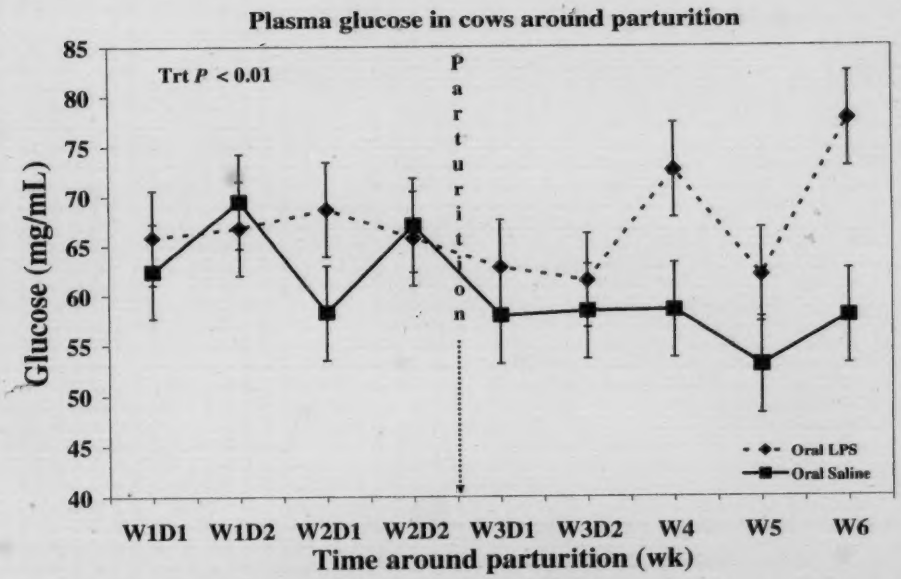

Figure 3.7. Concentration of glucose in the plasma of periparturient dairy cows treated orally with saline (control) or lipopolysaccharide (LPS) twice per week during -2 and -1 week before (W1 and W2) as well as 1 week after calving (W3). Blood samples also were collected once per week on week 2, 3, and 4 after calving (W4, W5, and W6).

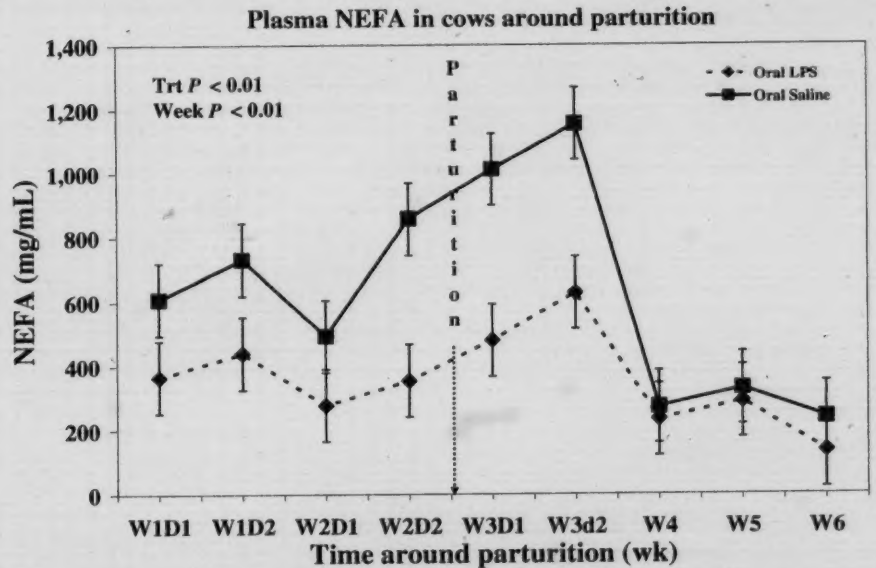

Figure 3.8. Concentration of non-esterified fatty acids in the plasma of periparturient dairy cows treated orally with saline (control) or lipopolysaccharide (LPS) twice per week during -2 and -1 week before (W1 and W2) as well as 1 week after calving (W3). Blood samples also were collected once per week on week 2, 3, and 4 after calving (W4, W5, and W6).

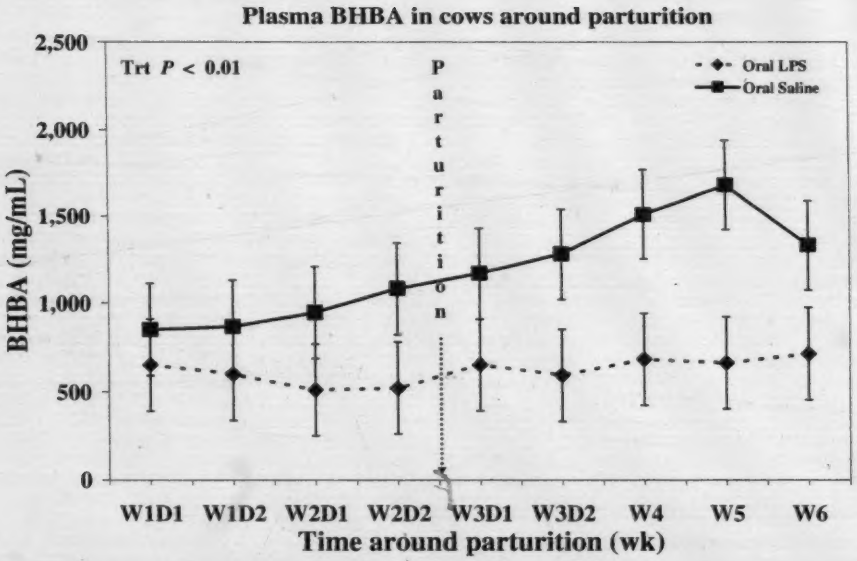

Figure 3.9. Concentration of β-hydroxy butyrate in the plasma of periparturient dairy cows treated orally with saline (control) or lipopolysaccharide (LPS) twice per week during -2 and -1 week before (W1 and W2) as well as 1 week after calving (W3). Blood samples also were collected once per week on week 2, 3, and 4 after calving (W4, W5, and W6).

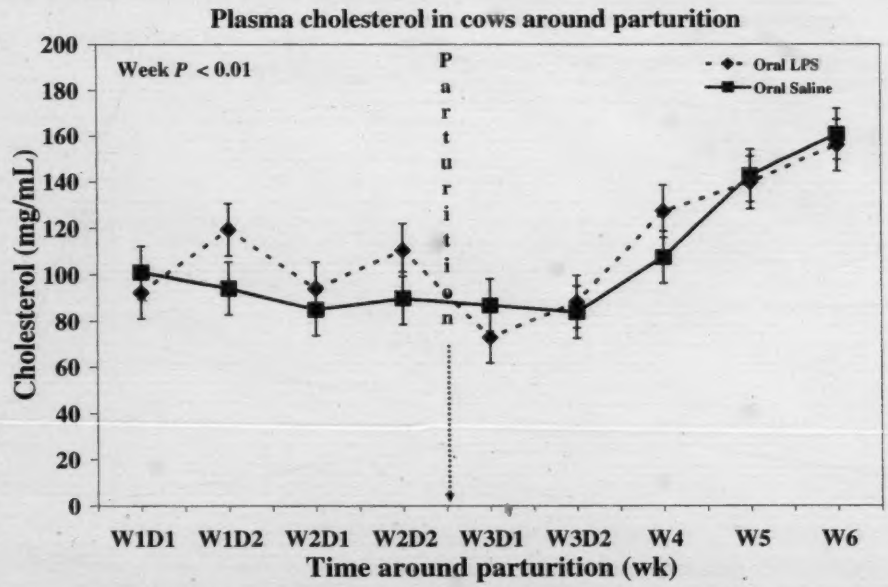

Figure 3.10. Concentration of cholesterol in the plasma of periparturient dairy cows treated orally with saline (control) or lipopolysaccharide (LPS) twice per week during -2 and -1 week before (W1 and W2) as well as 1 week after calving (W3). Blood samples also were collected once per week on week 2, 3, and 4 after calving (W4, W5, and W6).

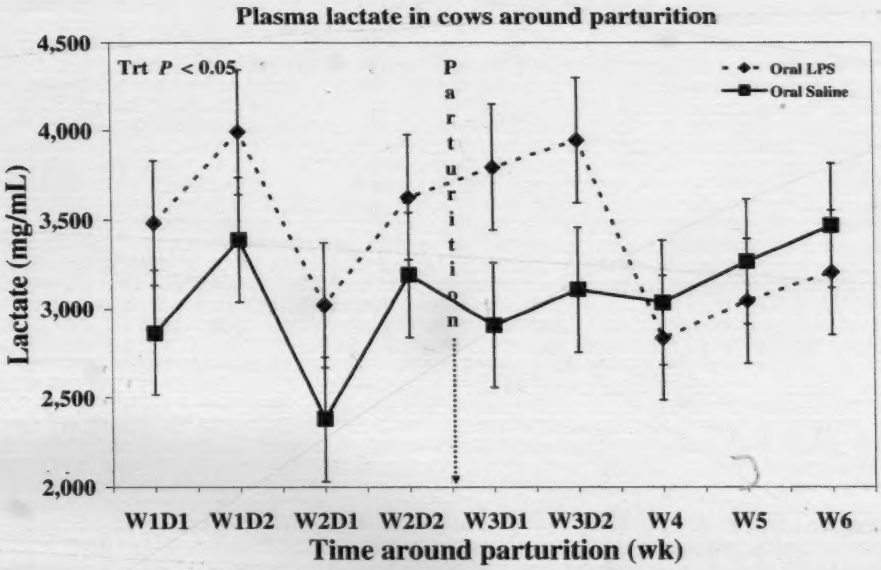

Figure 3.11. Concentration of lactate in the plasma of periparturient dairy cows treated orally with saline (control) or lipopolysaccharide (LPS) twice per week during -2 and -1 week before (W1 and W2) as well as 1 week after calving (W3). Blood samples also were collected once per week on week 2, 3, and 4 after calving (W4, W5, and W6).

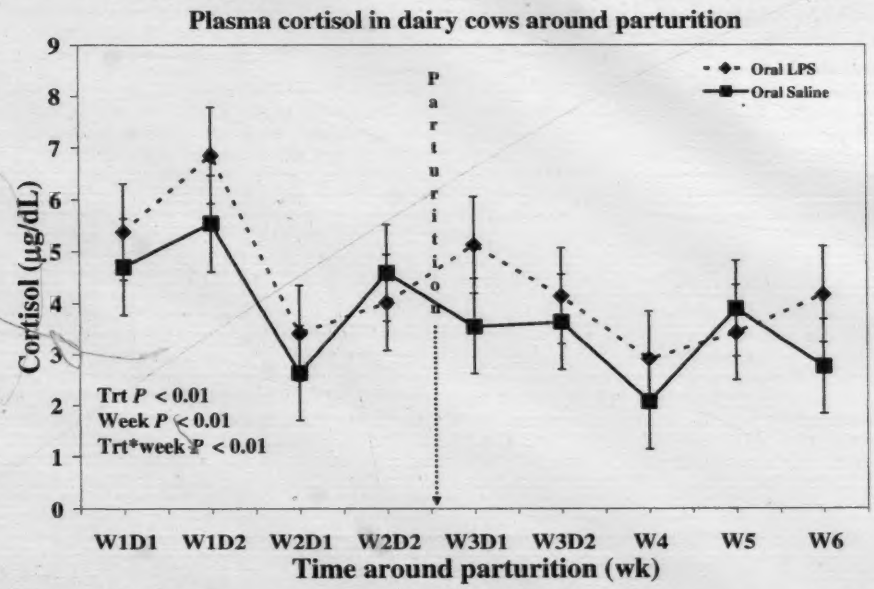

Figure 3.12. Concentration of cortisol in the plasma of periparturient dairy cows treated orally with saline (control) or lipopolysaccharide (LPS) twice per week during -2 and -1 week before (W1 and W2) as well as 1 week after calving (W3). Blood samples also were collected once per week on week 2, 3, and 4 after calving (W4, W5, and W6).

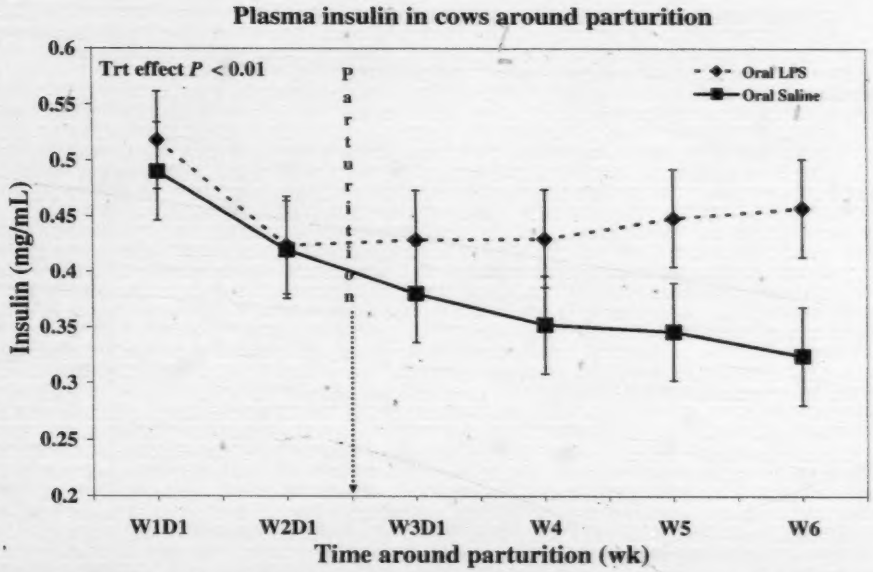

Figure 3.13. Concentration of insulin in the plasma of periparturient dairy cows treated orally with saline (control) or lipopolysaccharide (LPS) twice per week during -2 and -1 week before (W1 and W2) as well as 1 week after calving (W3). Blood samples also were collected once per week on week 2, 3, and 4 after calving (W4, W5, and W6).

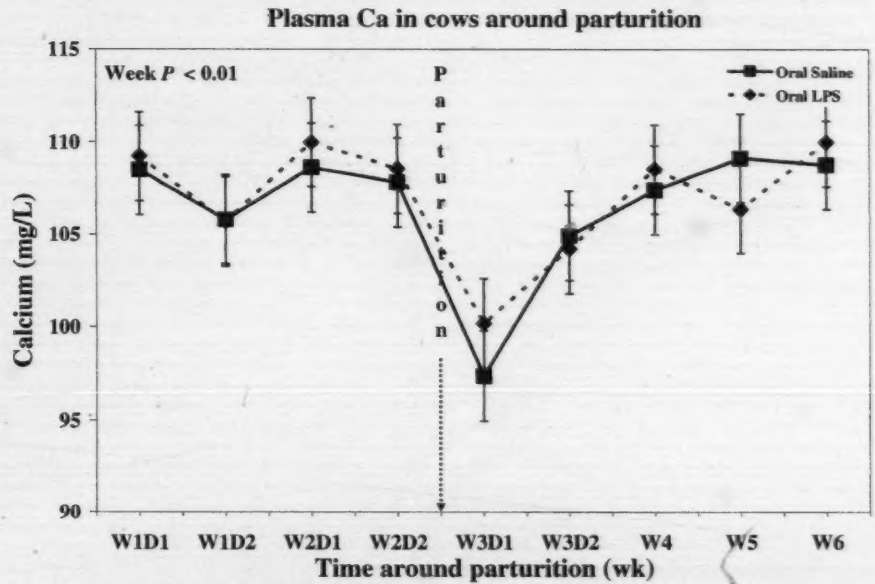

Figure 3.14. Concentration of calcium in the plasma of periparturient dairy cows treated orally with saline (control) or lipopolysaccharide (LPS) twice per week during -2 and -1 week before (W1 and W2) as well as 1 week after calving (W3). Blood samples also were collected once per week on week 2, 3, and 4 after calving (W4, W5, and W6).

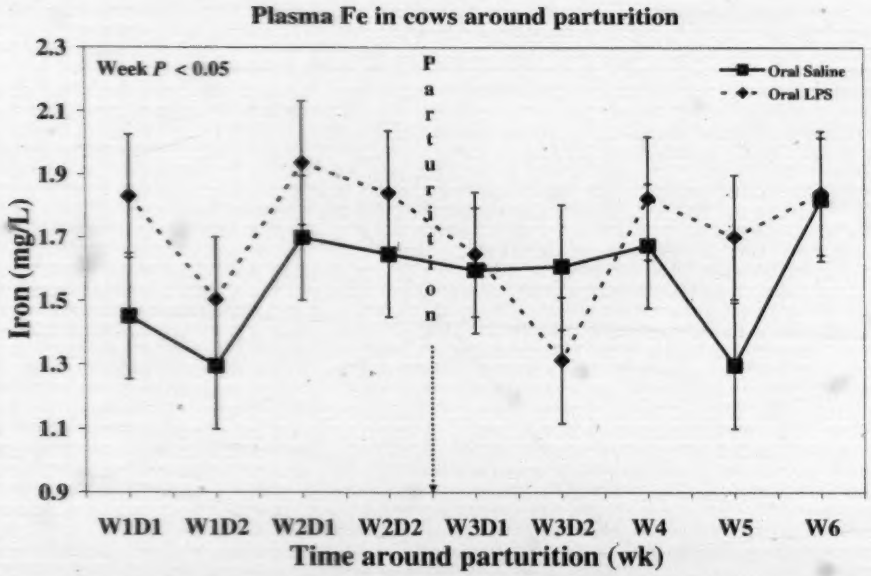

Figure 3.15. Concentration of iron in the plasma of periparturient dairy cows treated orally with saline (control) or lipopolysaccharide (LPS) twice per week during -2 and -1 week before (W1 and W2) as well as 1 week after calving (W3). Blood samples also were collected once per week on week 2, 3, and 4 after calving (W4, W5, and W6).

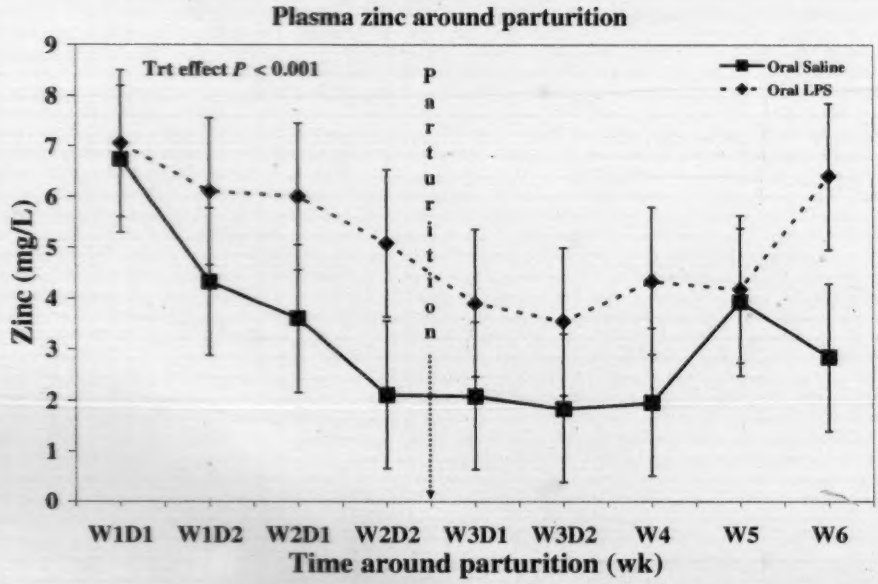

Figure 3.16. Concentration of zinc acids in the plasma of periparturient dairy cows treated orally with saline (control) or lipopolysaccharide (LPS) twice per week during -2 and -1 week before (W1 and W2) as well as 1 week after calving (W3). Blood samples also were collected once per week on week 2, 3, and 4 after calving (W4, W5, and W6).

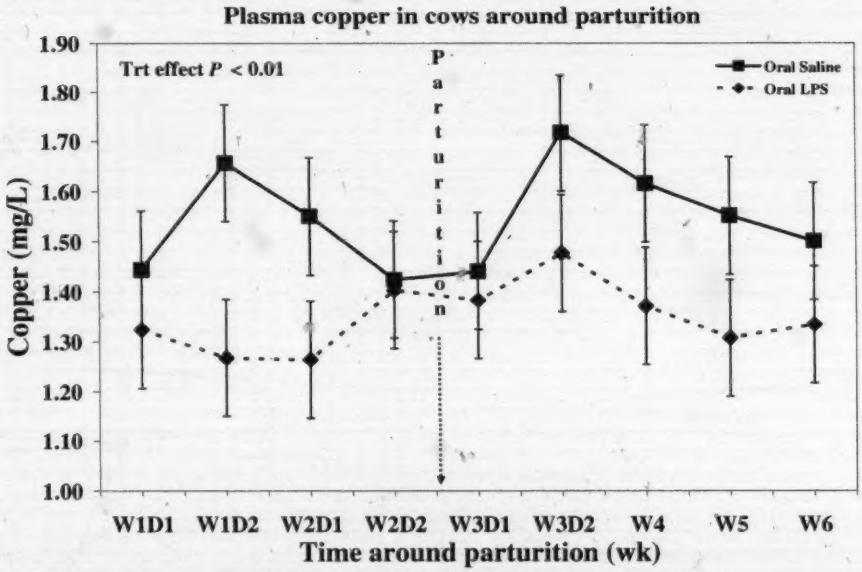

Figure 3.17. Concentration of copper in the plasma of periparturient dairy cows treated orally with saline (control) or lipopolysaccharide (LPS) twice per week during -2 and -1 week before (W1 and W2) as well as 1 week after calving (W3). Blood samples also were collected once per week on week 2, 3, and 4 after calving (W4, W5, and W6).

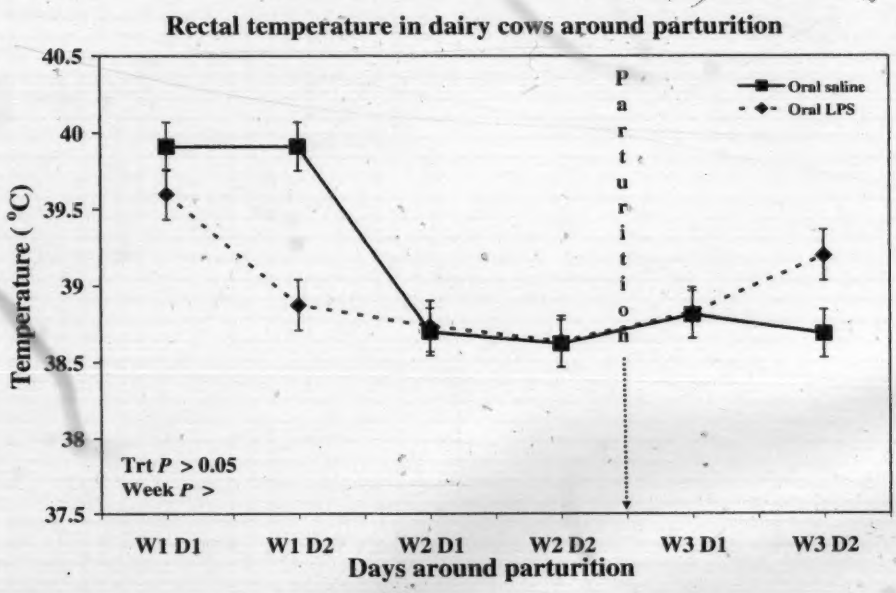

Figure 3.18. Rectal temperature in dairy cows treated orally with saline (control) or lipopolysaccharide (LPS) twice per week during -2 and -1 week before (W1 and W2) as well as 1 week after calving (W3).

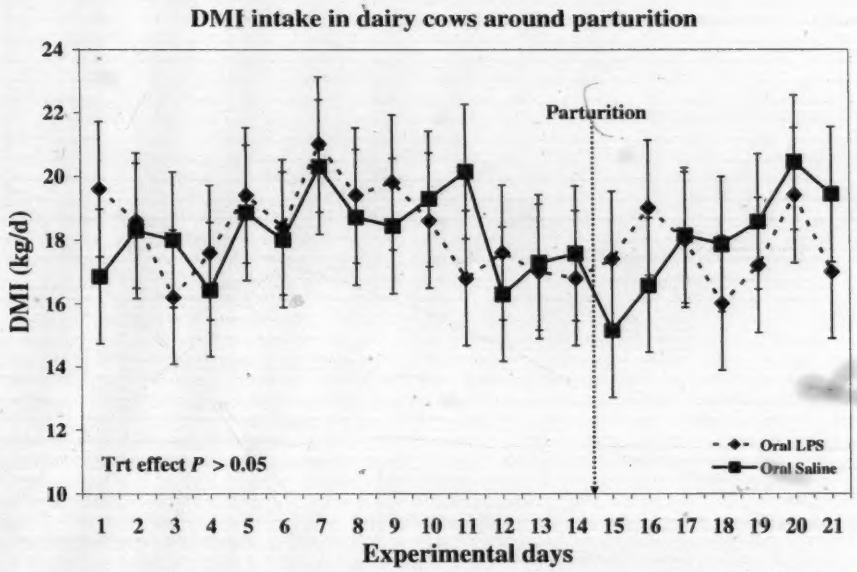

Figure 3.19. The dry matter intake of dairy cows treated with oral saline (control) and oral LPS (treatment) two weeks before and one week after parturition.

Table 3.1 Incidence of postpartal metabolic disorders in the two experimental groups sprayed orally with saline (control) or saline with lipopolysaccharide from *Escherichia coli* 0111:B4 (treatment) twice per wk during wk -2, and -1 before calving and 1 wk after calving.

Control Group Cow number	Clinical disease	Treatment group Cow number	Clinical disease
2360	Off feed 5 day, Temperature 40°C	2349	Normal
2113	Calf dead, Retained Placenta, Metritis	2369	Normal
2013	Twice Mastitis, Off feed, Temperature 39°C	2370	Lameness 31 d pp Mastitis 56 d pp
2163	Off feed 20 d post parturition, Fatty liver	2310	Normal
2412	Ketosis 3 d pp	2406	Normal
2168	Normal	2417	Normal
2268	Normal	2277	Normal
2371	Off feed, Treated with antibiotics	2423	Normal
307	Downer cow, culled		

Table 3.2. The differences in various parameters in cows administered either LPS or saline orally beginning 2 weeks and one week before parturition and one week after parturition.

Variables	Differences recorded between the LPS treated group and control group
I. Acute phase proteins	
1. Serum amyloid A	No significant difference
2. Lipopolysaccharide binding protein	No significant difference
3. Haptoglobin	No significant difference
II. Plasma immunoglobulins	
1. Immunoglobulin A	No significant difference
2. Immunoglobulin G	Lower concentration in treatment group
3. Immunoglobulin M	Higher concentration in treatment group
III. Plasma metabolites	
1. Non-esterified Fatty acids	Lower concentration in treatment group
2. Beta-hydroxy butyric acid	Lower concentration in treatment group
3. Glucose	Higher concentration in treatment group
4. Lactate	Higher concentration in treatment group
5. Insulin	Higher concentration in treatment group
6. Cholesterol	No significant difference
7. Cortisol	No significant difference
IV. Plasma minerals	
1. Calcium	No significant difference
2. Iron	No significant difference
3. Zinc	Higher concentration in treatment group
4. Copper	Lower concentration in treatment group
V. Rectal temperature	No significant difference
VI. Dry matter intake	No significant difference

Chapter 4

Increased Incidence of Metabolic Diseases and Alteration of Humoral Immune Responses during Intermittent Endotoxemia in Periparturient Dairy Cows

Abstract

Although endotoxin has been implicated as the causal agent of multiple metabolic diseases in dairy cattle, there is a lack of information regarding effects of chronic experimental endotoxemia in periparturient dairy cows. Therefore, the objective of this research was to evaluate the effects of repeated intravenous administration of LPS around parturition on metabolic, immune, clinical, and productive responses of periparturient dairy cows. Sixteen pregnant Holstein cows were infused twice per wk intravenously either saline (n = 8) or with three increasing dosages of LPS (n = 8) from *Escherichia coli* 0111:B4 at 0.01, 0.05, and 0.5 μg/kg BW during -2 and -1 wk before the anticipated day of parturition as well as during the first week after calving. Blood samples taken from the jugular vein were analyzed for serum amyloid A (SAA), lipopolysaccharide-binding protein (LBP), haptoglobin, anti-LPS immunoglobulin A (IgA), IgG, and IgM as well as cortisol, non-esterified fatty acids (NEFA), beta-hydroxybutyric acid (BHBA), glucose, lactate, insulin, cholesterol, calcium (Ca), iron (Fe), zinc (Zn), copper (Cu) in plasma. Also, rectal temperature, respiration rate, rumen contractions, dry matter intake (DMI), and milk production were measured. Results indicated that intermittent experimental endotoxemia was associated with greater incidence of metabolic and infectious diseases in transition dairy cows. Repeated infusion of LPS increased plasma concentrations of IgG and cortisol and lowered those of IgM and cholesterol as well as milk production in

transition dairy cows. Results also showed that plasma SAA, LBP, haptoglobin, IgA, NEFA, BHBA, glucose, lactate, insulin, Ca, Fe, Zn, Cu, rectal temperature, respiration rate, rumen contractions, and DMI were not affected by treatment. In conclusion, this study indicated that intermittent experimental endotoxemia increased incidence of metabolic and infectious diseases and diminished milk production in periparturient dairy cows. Administration of LPS also increased plasma concentrations of anti-LPS IgG and cortisol and lowered those of anti-LPS IgM and cholesterol but had no effect on other metabolic, immune, and clinical variables measured.

4.1 Introduction

A large body of evidence implicates endotoxin as a causal agent of multiple metabolic diseases in dairy cows. For example, Aiumlamai et al. (1992) indicated that endotoxin might be involved in the etiology of milk fever. In addition, Eades (1993; 1997) suggested involvement of endotoxin in suppression of reticulorumen motility and in the etiology of displaced abomasum. Moreover, Ametaj et al. (2005) showed a strong relationship between acute phase reactants to endotoxin and fatty liver in postpartal dairy cows. Multiple investigators have suggested a role for endotoxin in the etiology of laminitis (Boosman et al., 1991; Nocek, 1997). In addition, endotoxin has been shown to affect reproductive performance by modulating luteal functions and production of progesterone in dairy cows as well as by causing apoptosis of luteal cells (Grant et al., 2007; Mishra and Dhali, 2007). Recently, Andersen (2003) coined the term bovine endotoxicosis to describe negative effects of endotoxin and its relation to production diseases in dairy cows.

Another line of reasoning has provided evidence that repeated exposure to LPS is associated with tolerance to endotoxin. Tolerance to LPS has been induced by exposing naive animals to sublethal doses of LPS, resulting in a suppressed proinflammatory response to subsequent challenges with higher doses of LPS. The mechanism by which priming with sublethal doses of LPS causes development of tolerance to endotoxin is complex and not understood at present. Interestingly, Lehner et al. (2001) demonstrated that induction of LPS tolerance has positive effects on innate immune function, including increased neutrophils recruitment and increased number and activity of phagocytic cells. Moreover, Varma et al. (2005) reported that clearance of a *Pseudomonas* challenge was enhanced in LPS tolerant mice and could be augmented by neutralization of IL-10.

There are only a few studies reporting presence of endotoxin into the blood circulation of dairy cows (Andersen et al., 1994). The reason for that is that endotoxin is quickly removed from systemic circulation by liver hepatocytes as well as liver and spleen macrophages, even if administered intravenously in large amounts (Mathison and Ulevitch, 1979; Andersen et al., 1996; Shao et al., 2007). Interestingly, clearance of endotoxin from systemic circulation is affected by the health status of the host. For example, Andersen et al. (1996) reported measurement of plasma endotoxin only in the cows that spontaneously developed hepatic lipidosis but not in the healthy ones. The main sources of endotoxin in dairy cows are pathogenic Gram-negative bacteria present in the gastrointestinal tract as well as in postpartal infected uterus and mammary gland. Under pathological conditions, endotoxin translocates into the host blood circulation and causes multiple dysfunctions reflected by major shifts in the metabolic and immune

responses of the host. The mechanism by which endotoxin translocates into the host blood circulation is not known. Recently, Emmanuel at al. (2007) demonstrated, under Ussing chamber conditions, that LPS permeates through rumen and colon tissue walls if present in the solution at 500 μg/mL. This finding suggests that deactivating mechanisms might be present in the gastrointestinal tract to prevent endotoxin translocation and that only during presence of overwhelming amounts of endotoxin in the rumen fluid might endotoxin translocate into the host blood circulation. What happens to endotoxin after translocation is not clear. Whether endotoxin follows through the portal blood to be removed by Kupfer cells in the liver or enters into the lymphatic system and is cleared by lymph node macrophages again is not known.

A number of investigators have studied effects of single intravenous or intramammary administration of LPS in dairy cows in order to elucidate host metabolic, endocrine, and immune responses to endotoxin. However, to our best knowledge, there are only two studies involving repeated intravenous infusion of LPS in cattle but none in dairy cows. For example, Kahl and Elsasser (2006) showed that injection of testosterone modified host TNF-alpha production to two intravenous infusions of LPS. In addition, Bieniek et al. (1998) showed that two intravenous administrations of LPS, one week apart, in calves modified in vitro production of TNF-alpha.

Therefore, the purpose of the present study was to examine the effects of intermittent endotoxemia through repeated intravenous infusion of LPS from *Escherichia coli*

0111:B4 on the incidence of metabolic and infectious diseases as well as on metabolic, immune, mineral, and production responses in periparturient dairy cows.

4.2. Materials and methods

4.2.1. Animals and treatments. Sixteen clinically healthy heifers and multiparous Holstein cows nearing term were equally distributed to two different treatment groups of 8 animals each. Six cows from each group were match for age and parity, whereas two cows per group were slightly different in age and parity. Each group was assigned to one treatment either: 1) intravenous lipopolysaccharide (LPS) or 2) intravenous saline (control). Administration of LPS was performed twice per week during 2 weeks before the anticipated day of parturition and 1 week after parturition in weekly increasing doses of 0.01 μg, 0.05 μg, and 0.1 μg/kg BW on day 1 and 3 of each week. The doses of 0.01, 0.05, and 0.1 μg/kg BW of LPS were chosen from previous experiments demonstrating effects of different doses of LPS in various categories of cattle (Luthman et al., 1988; Aiumlamai and Kindhal, 1990; Giri et al., 1990; Gerros et al., 1996; Ohtsuka et al., 1997; Horadagoda et al., 2002; Jacobsen et al., 2003). The initial crystalline *Escherichia coli* LPS (Lipopolysaccharide-FITC from *Escherichia coli* strain 0111:B4 supplied by Sigma-Aldrich Canada Ltd.) containing 10 mg of purified LPS was dissolved in 10 mL of distilled water as suggested by the manufacturer and stored in the refrigerator. Before intravenous infusion, the injection area was shaved and aseptically washed and sterilized with iodine and 70% ethyl alcohol and the catheter was introduced into the jugular vein after proper restraint of the cows. The catheter (BD Angiocath 14 GA 5.25 IN 2.1 x 133 mm) was fixed 1h prior to initiation of infusion. The proper amount of LPS was mixed in

100 mL of saline and was infused by dripping into the jugular vein by the catheter. The saline or LPS saline was introduced at a rate of 2 mL/min.

All cows were restrained to take blood samples from jugular vein twice per week during -2 and -1 wk before the anticipated day of parturition and 1 wk after parturition as well as once per wk during wk 2, 3, and 4 after parturition. Blood samples were collected minutes before administration of treatment in Na-EDTA tubes and put immediately in ice, and centrifuged within 20 minutes (Rotanta 460R, Hettich Zentrifugan, Tuttlingen, Germany) at 3000*g for 20 min at 4°C. The plasma was separated and stored at -20°C until analyses.

4.2.2. Monitoring of animals for clinical parameters. Rectal temperature, respiratory rate, and rumen contractions were measured before treatment as well as every hour, up to 6 hours after administration of treatment. Feed intake was recorded daily during the entire experimental period. All disease and medication history was recorded for each cow throughout the entire experimental period.

4.3. Sample analysis

The analyses of various plasma variables was performed using the same standard procedures as described in chapter 3.3.

4.4. Statistical analysis

The MIXED procedure of SAS (SAS Institute Inc., Cary, NC, USA Version 9.1.3) was used to analyze the blood variables. The repeated measures design was used by using the following model:

$\mathbf{Y = \mu + T_i + W_j + TW_{ij} + e_{ijkl}}$ where **T** = treatment, **W** = week, **TW** = Interaction of treatment and week, $\mathbf{e_{ijkl}}$ = random error.

The PDIFF option was used to compare the LS means. Measurements of same animal were considered as repeated measures. The cow within **T x W** was used as the experimental unit. The covariance structure of the repeated measurements for each variable was modeled separately according to the lowest values of fit statistics based on the BIC (Bayesian information criteria). The significance of results was declared at $P < 0.05$.

4.5. Results

4.5.1. Acute phase proteins.

Serum amyloid A. There was no difference in the plasma concentration of SAA ($P > 0.05$; Figure 4.1) between the treatment group and the control group. However, wk affected plasma concentration of SAA ($P < 0.05$). The concentration of SAA was high in both groups of cows around parturition and then declined to lower levels starting from wk 2 until wk 6 after parturition. No interaction between wk and treatment was evidenced ($P > 0.05$).

Lipopolysaccharide binding protein. Plasma concentration of LBP was not different between the intravenous (iv) LPS group and the iv saline group ($P > 0.05$; Figure 4.2). On the other hand, wk affected plasma concentrations of LBP with greater concentrations one wk before calving and lowest level on wk 5 after calving ($P < 0.05$). Although concentration of LBP in the saline treated group was greater than in the treatment group (88,224 vs 56,818 ng/mL) the difference did not reach significance. There was no wk by treatment interaction evidenced for plasma LBP ($P > 0.05$).

Haptoglobin. Plasma concentration of haptoglobin was not different between the two treatment groups ($P > 0.05$; Figure 4.3). Similarly with plasma LBP, haptoglobin in the LPS group was numerically greater when compared to the control group; however, the difference did not reach significance. Plasma haptoglobin was greater the wk before and after parturition and declined thereafter reaching the lowest concentration during wk 5 and 6 after calving. No evidence for interaction between wk and treatment was obtained for plasma haptoglobin ($P > 0.05$).

4.5.2. Plasma immunoglobulins

Immunoglobulin A. Plasma IgA specific against LPS was not different between the treatment groups ($P > 0.05$; Figure 4.4). Also, no effect of wk or treatment by wk interaction was obtained ($P > 0.05$). Plasma IgA remained unchanged related to parturition except for a steep increase on wk 4 after calving in the LPS treated group.

Immunoglobulin G. Concentration of IgG specific against LPS was greater in the LPS treated group compared to the saline group ($P < 0.05$; Figure 4.5) starting from wk 2 and up to wk 4 of the experimental period. However no wk effect or wk by treatment interaction was obtained for plasma IgG ($P > 0.05$ and $P > 0.05$, respectively).

Immunoglobulin M. Data showed a difference between the concentration of IgM in the plasma of the LPS treated group versus the saline group ($P < 0.01$; Figure 4.6). However, differently from plasma IgG, concentration of IgM was lower in the treated group compared to the control group. On the other hand, there was no wk effect or wk by treatment interaction for plasma IgM ($P > 0.05$ and $P > 0.05$, respectively). In fact, plasma IgM in the saline group was twice greater compared to the LPS treated group during the whole transition period (0.02 vs 0.0076 MMU/mL).

Plasma cortisol. Plasma cortisol was continuously greater in cows treated with LPS starting from wk 1 before parturition up to wk 4 after calving ($P < 0.001$; Figure 4.7). Also, data indicated a wk effect regarding plasma cortisol with concentration of cortisol falling sharply 2 wk before calving and remaining at around 4 μg/dL until 4 wk postpartum ($P < 0.05$). No treatment by week effect was obtained for plasma cortisol ($P > 0.05$).

4.5.3. Plasma metabolites.

Non-esterified fatty acids. No treatment effect was obtained for plasma NEFA ($P > 0.05$; Figure 4.8). However, there was a wk effect ($P < 0.05$) between the groups. Plasma

NEFA increased steeply increased in both groups during the first wk after parturition and then declined sharply reaching the lowest levels at 4 wk postpartum. No treatment by wk interaction was evidenced for plasma NEFA ($P > 0.05$).

Beta-hydroxy butyric acid. There was no significant difference in the concentration of BHBA in plasma between the LPS group and the saline group ($P > 0.05$; Figure 4.9). Plasma BHBA increased linearly from the lowest concentrations 2 wk before calving to the highest values 4 wk postpartum ($P < 0.05$). However, no wk by treatment effect was obtained for plasma BHBA ($P > 0.05$).

Plasma glucose. Plasma glucose was not different between the treatment groups ($P > 0.05$; Figure 4.10). Also, there was no difference related to wk or wk by treatment interaction regarding plasma glucose ($P > 0.05$). Cows treated with LPS had greater plasma glucose at two time points before and after parturition.

Lactate. There was no treatment effect ($P > 0.05$) or wk by treatment effect ($P > 0.05$) with respect to plasma lactate concentration (Figure 4.11). However, data indicated a wk effect regarding plasma lactate ($P < 0.001$). Lactate concentration increased 1 wk after parturition reaching to 5,000 mg/mL and then declined to lower than prepartal values at around 3,500 mg/mL during wk 4 after parturition. In the group of cows treated with LPS dropped to 1199 mg/mL on wk 3 after calving.

Plasma insulin. There was no treatment effect, wk effect, or treatment by wk interaction with respect to plasma insulin ($P > 0.05$, $P > 0.05$ and $P > 0.05$, respectively; Figure 4.12). Interestingly, plasma insulin was lower in cows treated with LPS than the control group during the whole experimental period; however, the difference did not reach significant level.

Cholesterol. Cows treated with LPS had lower plasma cholesterol versus saline group ($P < 0.001$; Figure 4.13). Also, a wk effect as well as a wk by treatment interaction was evidenced for plasma cholesterol ($P < 0.01$ and $P < 0.05$, respectively).

4.5.4. Plasma minerals.

Calcium and iron. Although both plasma Ca and Fe were lower in the group of cows treated with LPS, the differences did not reach significance ($P > 0.05$ and $P > 0.05$, respectively; Figures 4.14 and 4.15). Data showed a wk effect regarding plasma Ca^{2+} ($P < 0.01$); however, no such an effect was obtained for plasma Fe ($P > 0.05$). Concentration of Ca^{2+} declined during the wk before and after parturition and increased thereafter reaching prepartal levels 4 wk after calving. No such an effect was observed for plasma Fe^{2+}. Also, no wk by treatment interaction was obtained for both plasma Ca^{2+} and Fe^{2+} ($P > 0.05$ and $P > 0.05$, respectively).

Zinc and copper. Both plasma Zn^{2+} and Cu^{2+} were not affected by treatments administered to the two groups of cows ($P > 0.05$ and $P > 0.05$, respectively; Figures 4.16 and 4.17). There was a wk effect with regards to plasma Zn^{2+} ($P < 0.05$); however,

no such effect was obtained for plasma Cu ($P > 0.05$). Plasma Zn^{2+} declined from the highest values 2 wk before calving to the lowest values the wk before and the 2 wk following calving to increase slightly at 2 and 3 wk postpartum. Plasma Cu^{2+} increased after calving and was higher in the LPS treated group however, no significance was reached ($P > 0.05$).

Rectal temperature, respiration rate, and rumen contractions. There was no treatment effect ($P > 0.05$) or week effect ($P > 0.05$) with respect to rectal temperature throughout the entire experimental period (Figure 4.18). Also, there was no week by treatment effect evidenced for rectal temperature ($P > 0.05$). In addition, no differences in respiration rate or the number of rumen contractions per minute (data not shown) were obtained.

Dry matter intake. There was no treatment effect on dry matter intake between the group of cows treated with LPS and those treated with saline ($P > 0.05$; Figure 4.19). Also, there was no day effect or day by treatment interaction on DMI ($P > 0.05$ and $P > 0.05$, respectively).

4.6. Discussion

The main objectives of this study were to evaluate health status of periparturient dairy cows under intermittent endotoxemia and to assess whether induction of tolerance to endotoxin through repeated exposure to LPS would affect disease incidence in transition dairy cows. Systemic endotoxemia has been linked to a number of metabolic and

infectious diseases in dairy cattle including fatty liver, coliform mastitis, ruminal acidosis, laminitis, and displaced abomasum (Boosman et al., 1991; Andersen, 1994; Blum et al., 2000; Ametaj et al., 2005). Research conducted in other animal species has demonstrated that repeated systemic exposure to LPS induces tolerance to endotoxin and improves bacterial clearance and survival during bacterial challenge (Varma et al., 2005). However, to our best knowledge, no such studies have been reported in transition dairy cows and no investigations have been undertaken to examine metabolic, immune, clinical, and productive responses of dairy cows under intermittent experimental endotoxemia.

An interesting finding of our investigation is that intermittent endotoxemia was associated with greater incidence of metabolic and infectious diseases in peripartal dairy cows (Table 4.1). These data are contrary to the postulate that induction of tolerance by repeated exposure to LPS might prevent endotoxin-related metabolic and infectious diseases in dairy cattle. In fact, our findings are consistent with and expand previous suggestions implicating endotoxin as a causal agent of multiple metabolic and infectious diseases in transition dairy cows (Boosman et al., 1991; Andersen, 1994; Blum et al., 2000; Ametaj et al., 2005). The mechanism(s) by which endotoxin increased susceptibility of dairy cows to the above diseases is not clear at present; however, LPS has been shown to suppress adaptive immune responses and disturb multiple plasma metabolites and minerals in dairy cows (Bryn et al., 2007; Waldron, et al., 2003a, 2003b; Jacobsen et al., 2004, 2005). Therefore, our data do not support the line of thinking that

induction of tolerance to LPS by multiple intravenous infusions could be used as a preventive intervention against endotoxin-related diseases in peripartal dairy cows.

There was no effect of LPS on feed intake in our study. Previous research demonstrated that single intravenous infusion of LPS causes a state of anorexia and diminished feed intake in cattle (Steiger et al., 1999; Waldron et al., 2003a). Endotoxin exerts its anorexic effects indirectly through stimulation of IL-1 production in the hypothalamus (Dantzer et al., 1996; Plata-Salaman et al., 1998). Interleukin-1 is the key cytokine for development of centrally mediated signs of sickness during endotoxemia, including depression of food intake and food-motivated behavior (Dantzer et al., 1996; Plata-Salaman et al., 1998). The lack of effect of multiple blood infusions of LPS on feed intake in our case may be due to development of tolerance to LPS. During tolerance to LPS all pro-inflammatory cytokines including IL-1 are suppressed (Chen et al. (2005). In support of this line of thinking is the research work conducted by Shuster et al. (1991c) who demonstrated that the initial infusion of LPS in the mammary gland of dairy cows induced an acute phase response with systemic involvement. However, subsequent infusions failed to induce the systemic responses.

Intermittent endotoxemia had no affect on plasma concentration of three main acute phase proteins SAA, LBP, and haptoglobin under our experimental conditions. Previous research has shown that single intravenous infusion of LPS in dairy cows induces, within minutes, the release of cytokines like IL-1, IL-6, and TNF-α that stimulate liver production and release of SAA, LBP, and haptoglobin (Boosman et al., 1989; Werling et

al., 1996; Bannerman et al., 2003; Jacobsen et al., 2004). Development of tolerance to LPS in our case might explain the observed results. Indeed, in support of our reasoning is the report by Chen et al. (2005) who indicated tolerance to LPS diminished production of IL-1, IL-6, and TNF-α from macrophages necessary for stimulation of acute phase reactants from liver hepatocytes. Moreover, greater plasma cortisol in LPS-treated cows might have contributed to suppression of cytokine production and, therefore, in inhibition of acute phase proteins measured.

Concentrations of NEFA, BHBA, glucose, lactate and insulin in plasma were not altered by administration of LPS in our experiment. Research conducted by other investigators has shown that single intravenous infusion of LPS is associated with decreased plasma glucose and BHBA and increased plasma lactate (Werling et al., 1996; Waldron et al., 2003a). Additionally, Steiger et al. (1999) reported a biphasic response of plasma NEFA to intravenous infusion of LPS in heifers. Moreover, Waldron et al. (2003a) demonstrated that single intravenous LPS increased plasma insulin within hours from administration. Our results support the line of evidence that repeated exposure to LPS induces tolerance to endotoxin and prevents metabolite responses to LPS.

In addition to the plasma metabolites discussed above, plasma concentrations of four main minerals Ca^{2+}, Fe^{2+}, Zn^{2+}, and Cu^{2+} were not influenced by systemic infusion of LPS in treated animals. The lack of effect of mineral responses to intravenous LPS is contrary to reports of decreased plasma Ca^{2+}, Fe^{2+}, and Zn^{2+} and increased plasma Cu^{2+} during single intravenous treatments with LPS (Waldron et al., 2003b). This finding

again supports our line of thinking that repeated infusion of LPS induced development of tolerance to endotoxin in cows in our experiment.

Repeated administration of LPS in our study increased plasma concentrations of anti-LPS IgG antibodies lowered those of anti-LPS IgM and had no effect on anti-LPS IgA. Enhanced IgG antibodies in cows exposed several times to LPS is consistent with the development of a secondary antibody response to repeated antigen presentation. The contrary is true for IgM antibodies that are more important during first exposure (i.e., primary humoral immune response) of the host to antigen. The lack of effect of repeated LPS administration on IgA antibodies is related to the fact that IgA antibodies are released more into the mucosal layers instead of blood circulation. In agreement with this postulate is research conducted by Orlans et al. (1978) who showed that IgA antibodies disappear quickly from blood to the bile (Nilsson et al., 1988). Additionally, Nilsson et al. (1988) demonstrated that anti-LPS IgA antibody producing B cells are located mainly in the gastrointestinal tract than in the mesenteric lymphnodes.

Dietary cholesterol plays an important role in response to LPS. Research conducted by Huang et al. (2007) demonstrated that feeding C57BL/6 mice high cholesterol diets for 4 weeks raised concentration of cholesterol in plasma and increased the lethal and inflammatory effects of LPS. Although the reason why high plasma cholesterol aggravates the severity of responses to LPS is not known, Huang et al. (2007) showed that plasma SAA and the receptors for endotoxin (i.e., CD14 and toll-like receptor-4) in the Kupffer cells were increased in mice fed high cholesterol diets. Additionally, Levels

et al. (2007) showed that during experimental endotoxemia in humans there was low total cholesterol as well as low content of cholesterol in three main lipoproteins (i.e., VLDL, LDL, and HDL) combined with high levels of IL-6, IL-8, C-reactive protein, and LBP. Results of our study showed lower cholesterol in the group of cows infused with LPS. This is in agreement with research conducted by other investigators and might help cows to ease harmful effects of intermittent endotoxemia.

To maintain physiological homeostasis in response to LPS, the neuroendocrine and immune systems interact at several different levels. The hypothalamic-pituitary-adrenal (HPA) axis is believed to play a key role in this process in synthesis and secretion of glucocorticoids, such as cortisol, from the adrenal cortex (Anisman et al., 1998). Production and release of cortisol is important because it limits the extent of the inflammatory response through its immunosuppressive activities, which include decreasing the expression of genes for the pro-inflammatory molecules (Beishuizen and Thijs, 2003). Indeed, data from our study indicated that concentration of cortisol in plasma of our LPS treated cows was greater during the whole experimental period. Our findings are in line with previous research demonstrating that LPS is able to stimulate secretion of cortisol from both cortical cells of the adrenal gland and HPA axis (Vakharia et al., 2002).

Our data also showed that intravenous LPS did not affect rectal temperature, respiration rate or rumen contraction. Other research has shown that single administration of LPS has been reported to result in fever (high temperature) as well as increased respiration rate

(i.e., tachypnea) and a decrease in the number of rumen contractions (Eades, 1997; Almeida et al., 1999; Jacobsen et al., 2005). [Note: In fact, rectal temperature was greater in cows treated with LPS versus controls from 1 to 4 h after each LPS treatment (data not shown). All blood analyses and clinical observation were collected before and up to 6 h post-administration of LPS (i.e., at -15 min before treatment as well as at 15, 30, 60, 120, 180, 240, 300, and 360 min after LPS administration into the jugular vein; however, inclusion of these data in this thesis is beyond the scope of this masters' degree]. The lack of long-term effect of LPS on clinical variables measured in our experiment is in agreement with other investigations demonstrating that repeated exposure to LPS induces tolerance to endotoxin. For example, Beeson, (1947) and Atkins, (1960) reported that repeated administration of LPS attenuated the febrile response. The mechanism by which repeated exposure to LPS induces fever tolerance was not understood until recently when Roth et al. (1994) and Almeida et al. (1999) indicated a role of nitric oxide in suppression of IL-6 and TNF-α production and release. While Eades (1997) observed hypomotility of reticulo-rumen during induced endotoxemia by single intravenous infusion of LPS rumen motility of cows in our experiment was not affected by treatment. The mechanism by which single administration of endotoxin suppresses reticulo-rumen motility involves excessive production of catecholamines by adrenal glands or sympathetic nervous system and stimulation of inhibitory α_2-adrenergic receptors (Eades, 1997). The lack of effect in our case deserves more research to understand the mechanism by which intermittent endotoxemia induces tolerance.

In summary, our study showed that cows under conditions of intermittent endotoxemia had greater incidence of metabolic and infectious diseases during postpartum period compared to cows infused with saline. Repeated exposure to LPS increased plasma anti-LPS IgG antibodies and cortisol and lowered anti-LPS IgM antibodies and cholesterol. In contrast to effects reported for single injections of LPS most of the metabolic, immune, and clinical variables measured in our study were not affected by administration of LPS. The lack of effect of multiple intravenous infusions of LPS on those variables might be related to development of tolerance to LPS. Further research is warranted to elucidate the mechanisms by which endotoxin elicits immune, metabolic, and clinical tolerance when administered more than one time into the blood circulation. In conclusion, intermittent exposure to endotoxin had deleterious effects on health of transition dairy cows. Further investigation is necessary to determine why endotoxin increases the incidence of postpartal metabolic and infectious diseases in dairy cows.

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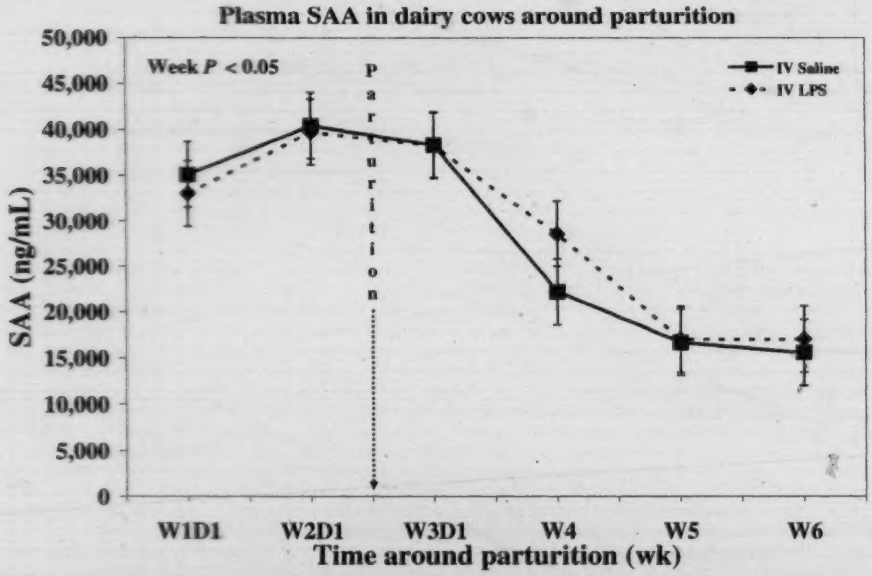

Figure 4.1. Concentration of SAA in the plasma of periparturient dairy cows treated intravenously with saline (control) or lipopolysaccharide (LPS) twice per week during -2 and -1 week before (W1 and W2) as well as 1 week after calving (W3). Blood samples also were collected once per week on week 2, 3, and 4 after calving (W4, W5, and W6).

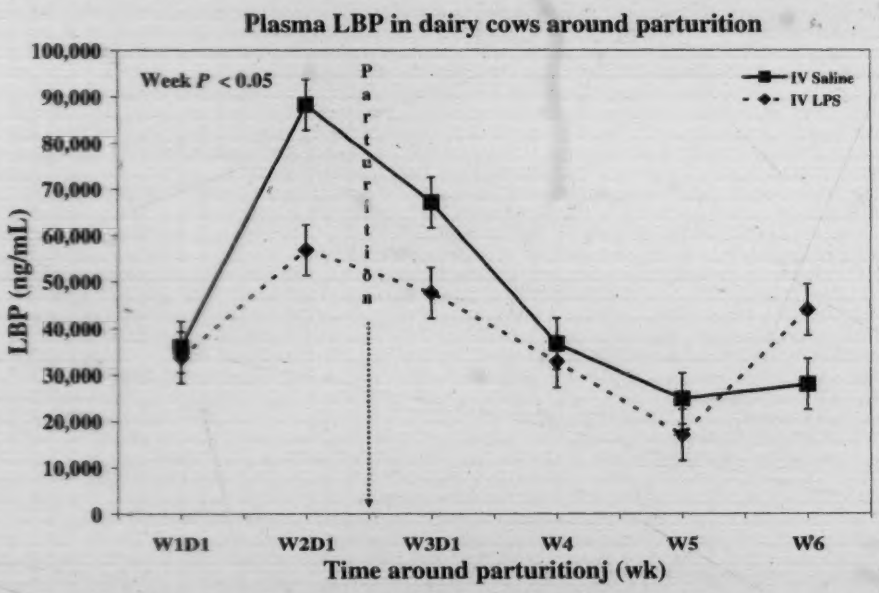

Figure 4.2. Concentration of LBP in the plasma of periparturient dairy cows treated intravenously with saline (control) or lipopolysaccharide (LPS) twice per week during -2 and -1 week before (W1 and W2) as well as 1 week after calving (W3). Blood samples also were collected once per week on week 2, 3, and 4 after calving (W4, W5, and W6).

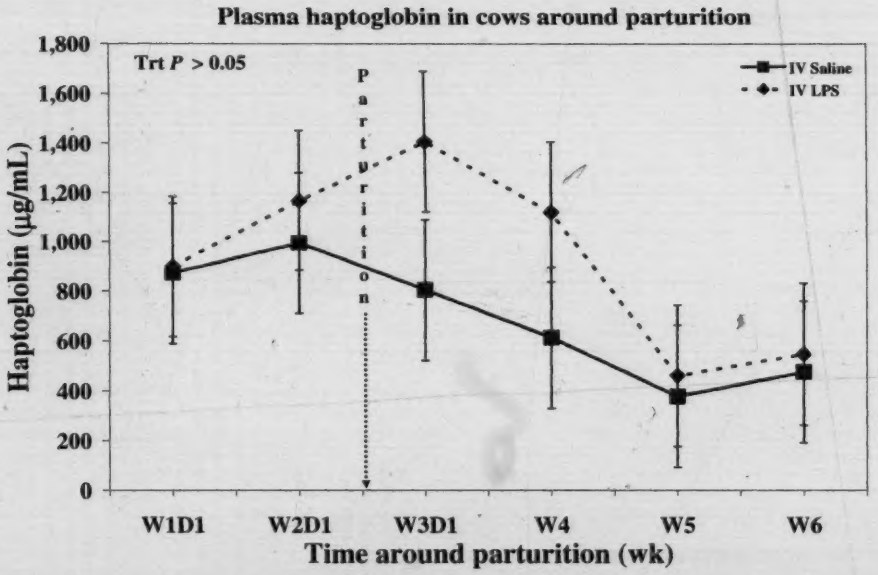

Figure 4.3. Concentration of haptoglobin in the plasma of periparturient dairy cows treated intravenously with saline (control) or lipopolysaccharide (LPS) twice per week during -2 and -1 week before (W1 and W2) as well as 1 week after calving (W3). Blood samples also were collected once per week on week 2, 3, and 4 after calving (W4, W5, and W6).

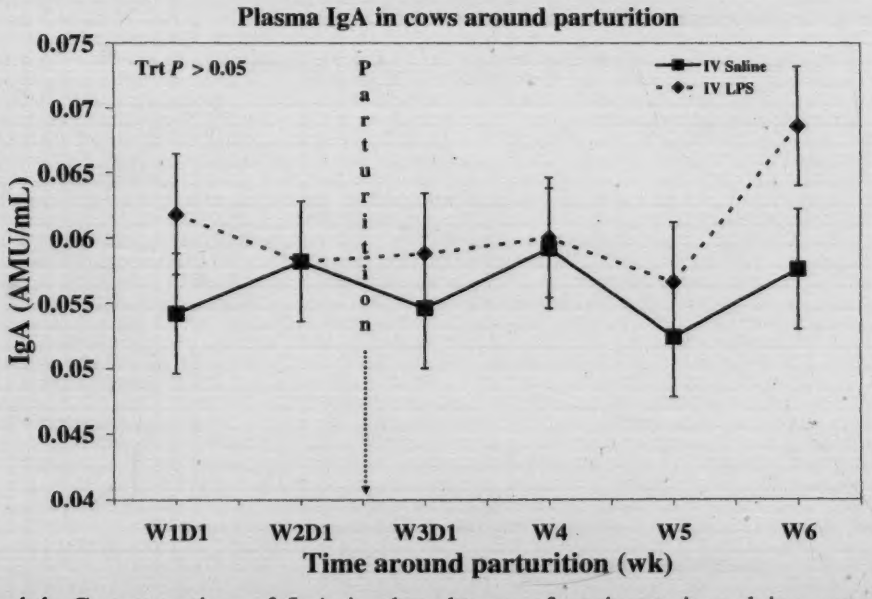

Figure 4.4. Concentration of IgA in the plasma of periparturient dairy cows treated intravenously with saline (control) or lipopolysaccharide (LPS) twice per week during -2 and -1 week before (W1 and W2) as well as 1 week after calving (W3). Blood samples also were collected once per week on week 2, 3, and 4 after calving (W4, W5, and W6).

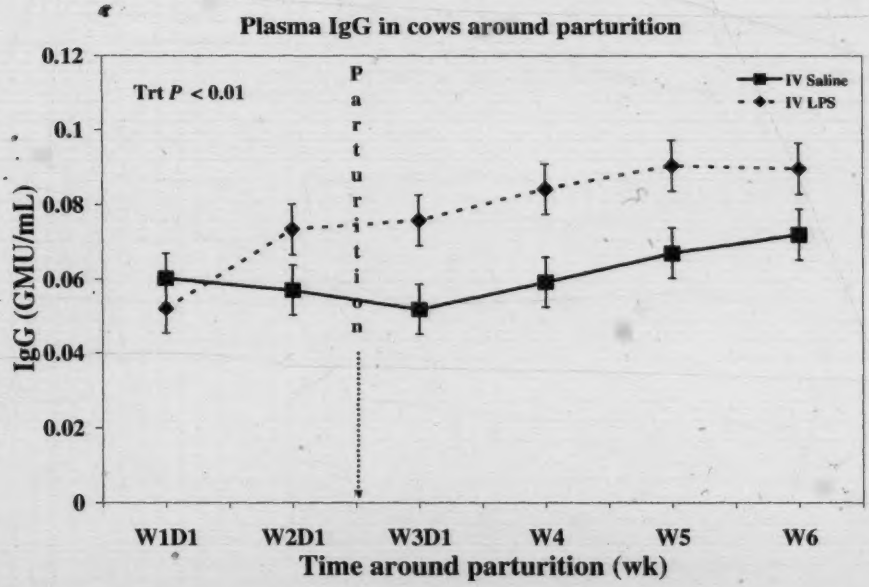

Figure 4.5. Concentration of IgG in the plasma of periparturient dairy cows treated intravenously with saline (control) or lipopolysaccharide (LPS) twice per week during -2 and -1 week before (W1 and W2) as well as 1 week after calving (W3). Blood samples also were collected once per week on week 2, 3, and 4 after calving (W4, W5, and W6).

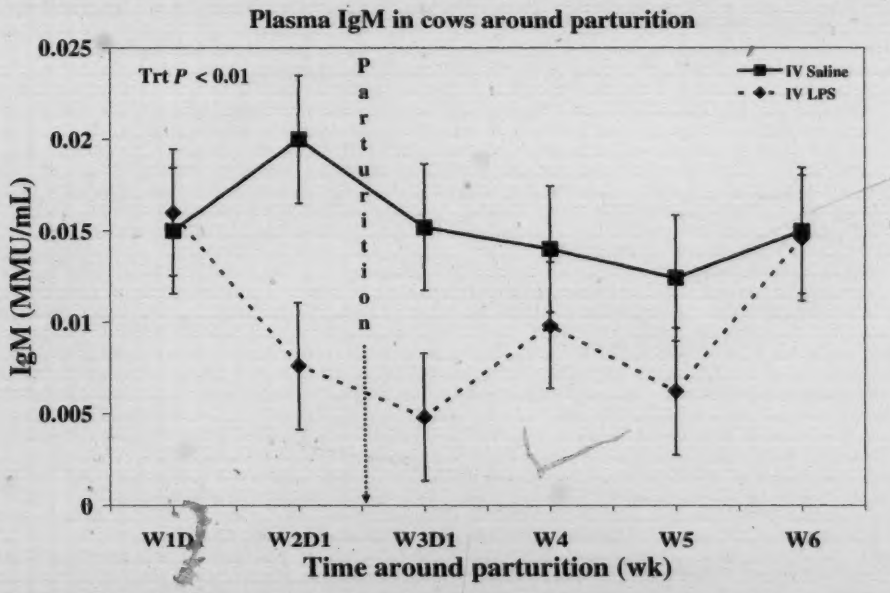

Figure 4.6. Concentration of IgM in the plasma of periparturient dairy cows treated intravenously with saline (control) or lipopolysaccharide (LPS) twice per week during -2 and -1 week before (W1 and W2) as well as 1 week after calving (W3). Blood samples also were collected once per week on week 2, 3, and 4 after calving (W4, W5, and W6).

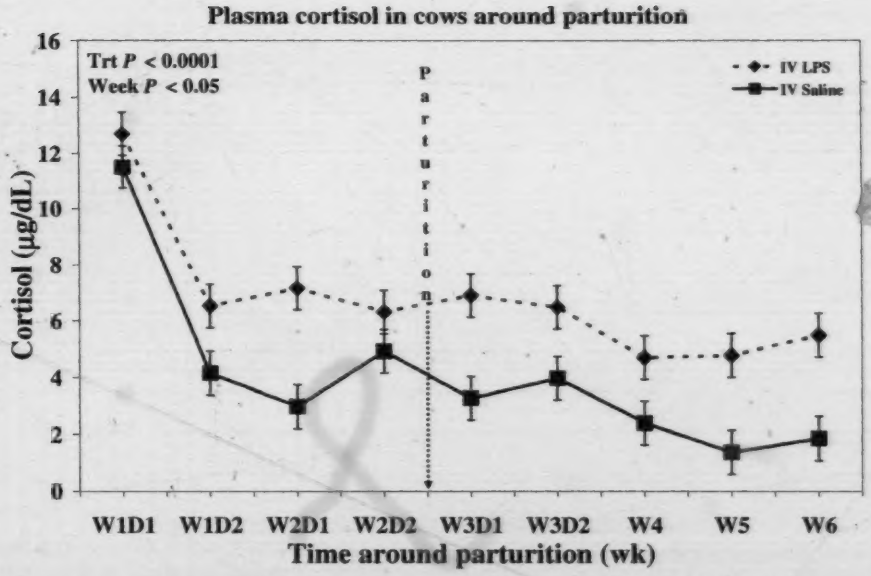

Figure 4.7. Concentration of cortisol in the plasma of periparturient dairy cows treated intravenously with saline (control) or lipopolysaccharide (LPS) twice per week during -2 and -1 week before (W1 and W2) as well as 1 week after calving (W3). Blood samples also were collected once per week on week 2, 3, and 4 after calving (W4, W5, and W6).

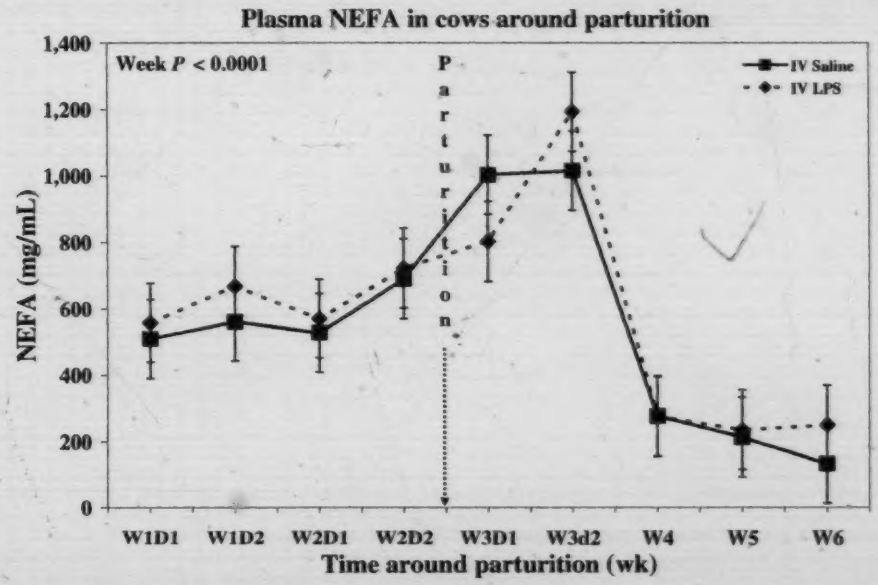

Figure 4.8. Concentration of non esterified fatty acids in the plasma of periparturient dairy cows treated intravenously with saline (control) or lipopolysaccharide (LPS) twice per week during -2 and -1 week before (W1 and W2) as well as 1 week after calving (W3). Blood samples also were collected once per week on week 2, 3, and 4 after calving (W4, W5, and W6).

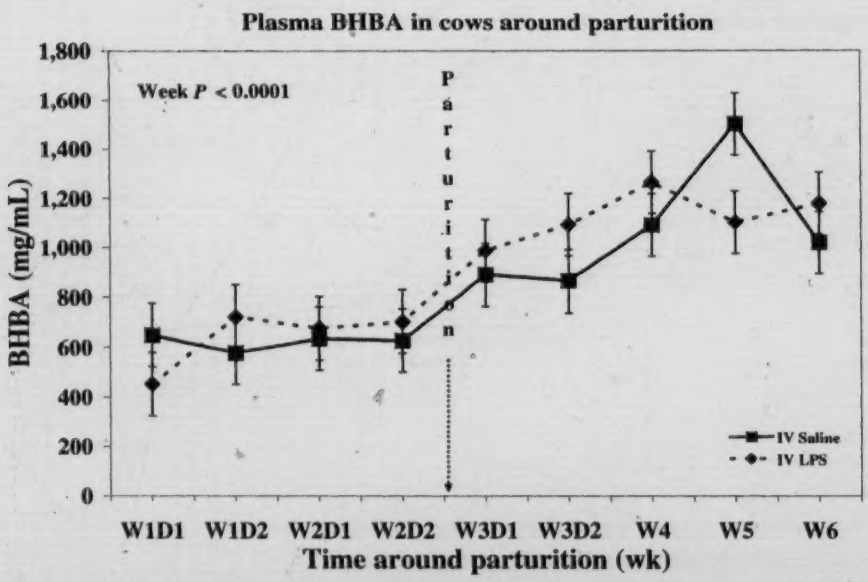

Figure 4.9. Concentration of β-hydroxy butyrate in the plasma of periparturient dairy cows treated intravenously with saline (control) or lipopolysaccharide (LPS) twice per week during -2 and -1 week before (W1 and W2) as well as 1 week after calving (W3). Blood samples also were collected once per week on week 2, 3, and 4 after calving (W4, W5, and W6).

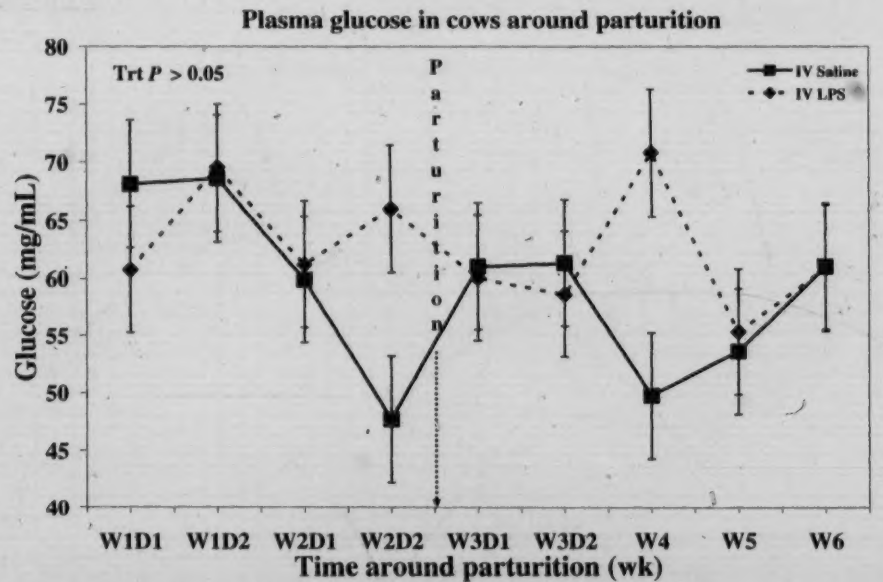

Figure 4.10. Concentration of glucose in the plasma of periparturient dairy cows treated intravenously with saline (control) or lipopolysaccharide (LPS) twice per week during -2 and -1 week before (W1 and W2) as well as 1 week after calving (W3). Blood samples also were collected once per week on week 2, 3, and 4 after calving (W4, W5, and W6).

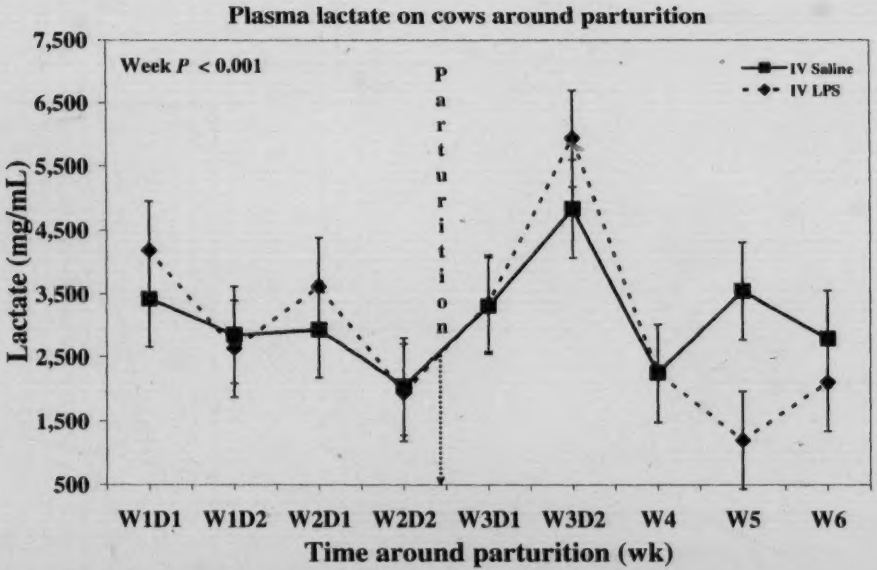

Figure 4.11. Concentration of lactate in the plasma of periparturient dairy cows treated intravenously with saline (control) or lipopolysaccharide (LPS) twice per week during -2 and -1 week before (W1 and W2) as well as 1 week after calving (W3). Blood samples also were collected once per week on week 2, 3, and 4 after calving (W4, W5, and W6).

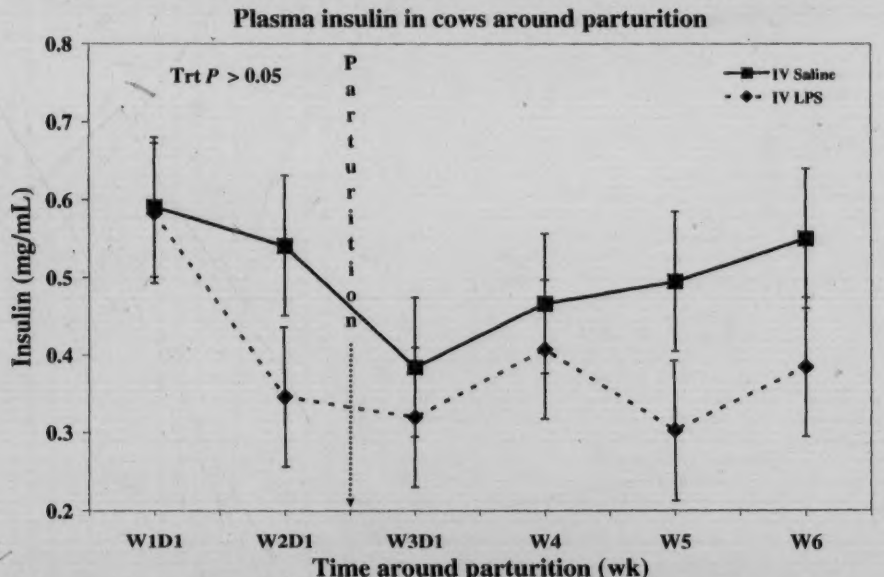

Figure 4.12. Concentration of insulin in the plasma of periparturient dairy cows treated intravenously with saline (control) or lipopolysaccharide (LPS) twice per week during -2 and -1 week before (W1 and W2) as well as 1 week after calving (W3). Blood samples also were collected once per week on week 2, 3, and 4 after calving (W4, W5, and W6).

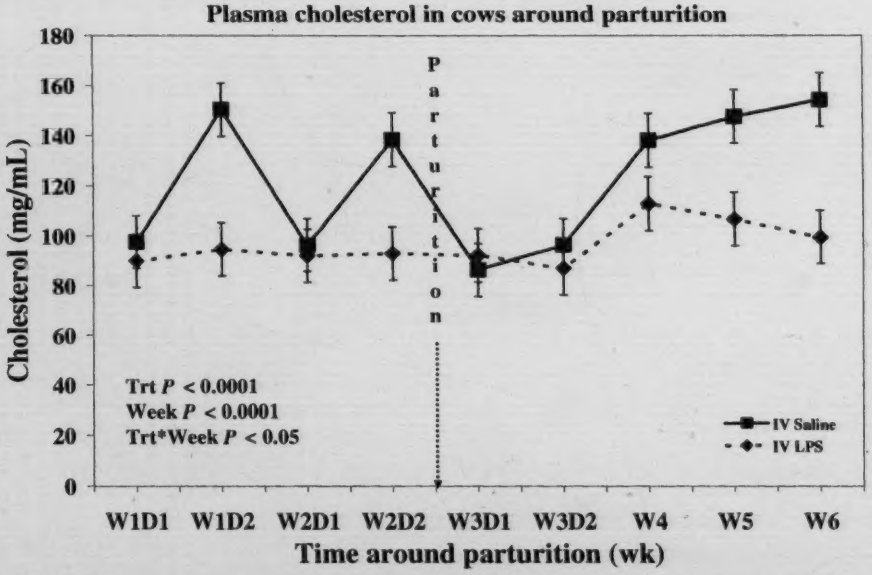

Figure 4.13. Concentration of cholesterol in the plasma of periparturient dairy cows treated intravenously with saline (control) or lipopolysaccharide (LPS) twice per week during -2 and -1 week before (W1 and W2) as well as 1 week after calving (W3). Blood samples also were collected once per week on week 2, 3, and 4 after calving (W4, W5, and W6).

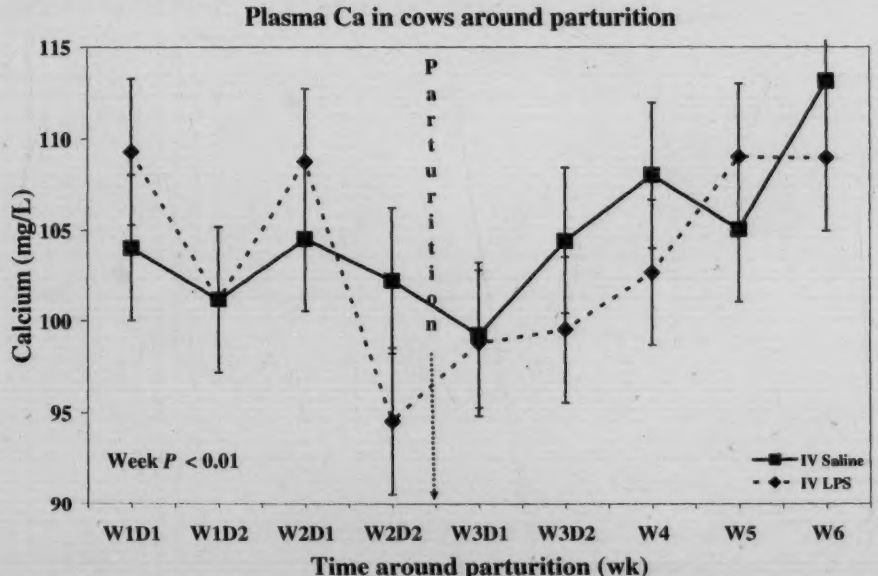

Figure 4.14. Concentration of calcium in the plasma of periparturient dairy cows treated intravenously with saline (control) or lipopolysaccharide (LPS) twice per week during -2 and -1 week before (W1 and W2) as well as 1 week after calving (W3). Blood samples also were collected once per week on week 2, 3, and 4 after calving (W4, W5, and W6).

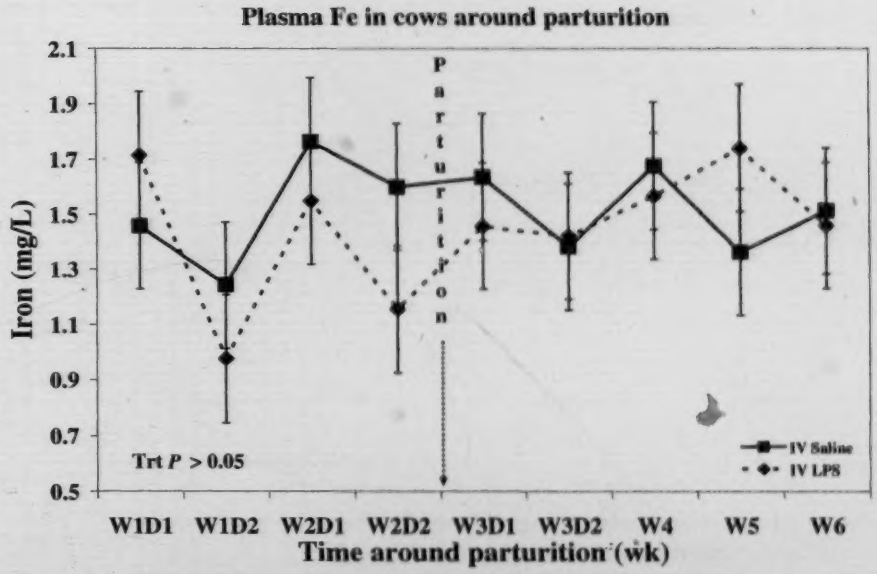

Figure 4.15. Concentration of iron in the plasma of periparturient dairy cows treated intravenously with saline (control) or lipopolysaccharide (LPS) twice per week during -2 and -1 week before (W1 and W2) as well as 1 week after calving (W3). Blood samples also were collected once per week on week 2, 3, and 4 after calving (W4, W5, and W6).

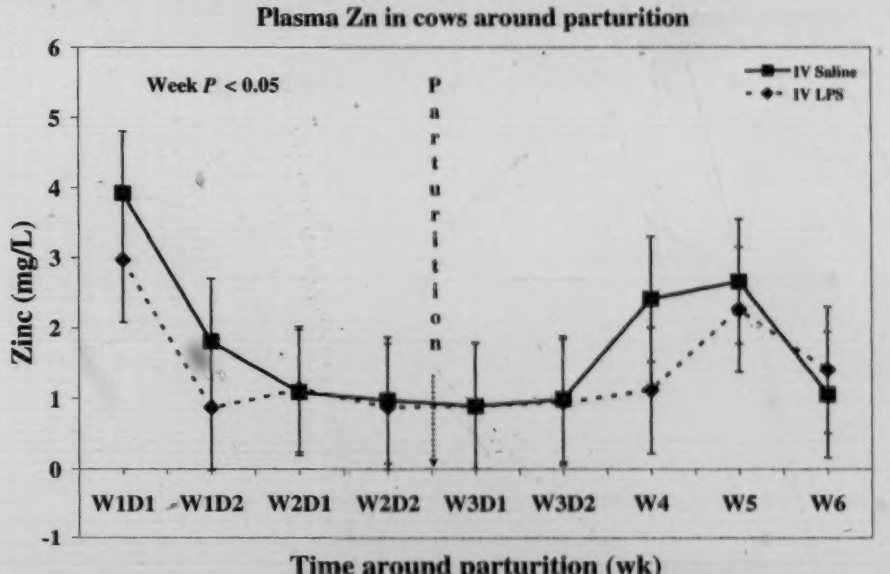

Figure 4.16. Concentration of zinc in the plasma of periparturient dairy cows treated intravenously with saline (control) or lipopolysaccharide (LPS) twice per week during -2 and -1 week before (W1 and W2) as well as 1 week after calving (W3). Blood samples also were collected once per week on week 2, 3, and 4 after calving (W4, W5, and W6).

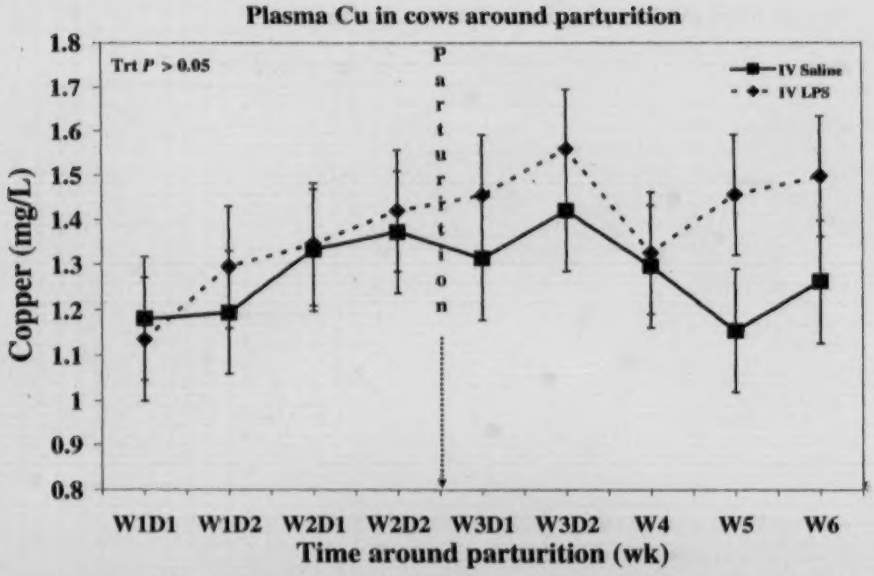

Figure 4.17. Concentration of copper in the plasma of periparturient dairy cows treated intravenously with saline (control) or lipopolysaccharide (LPS) twice per week during -2 and -1 week before (W1 and W2) as well as 1 week after calving (W3). Blood samples also were collected once per week on week 2, 3, and 4 after calving (W4, W5, and W6).

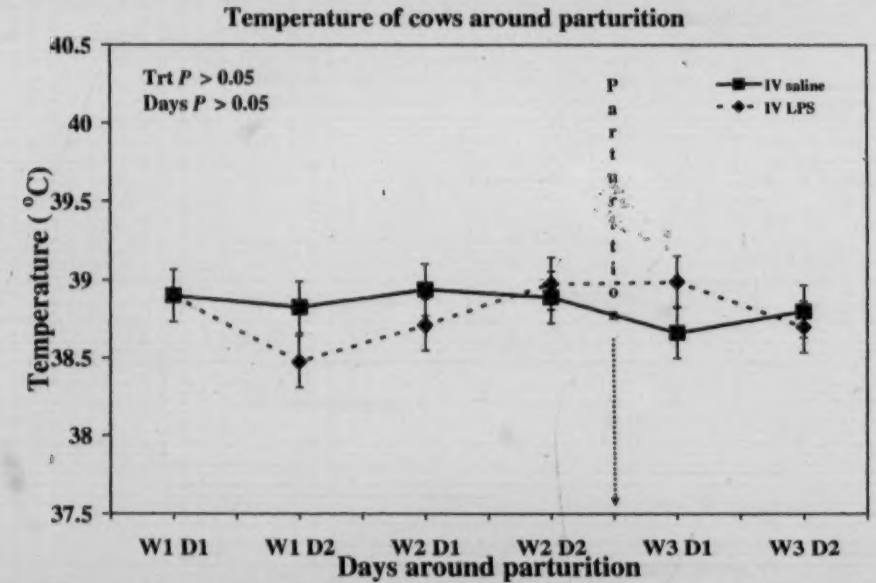

Figure 4.18. Rectal temperature of periparturient dairy cows treated orally with saline (control) or lipopolysaccharide (LPS) twice per week during -2 and -1 week before (W1 and W2) as well as 1 week after calving (W3).

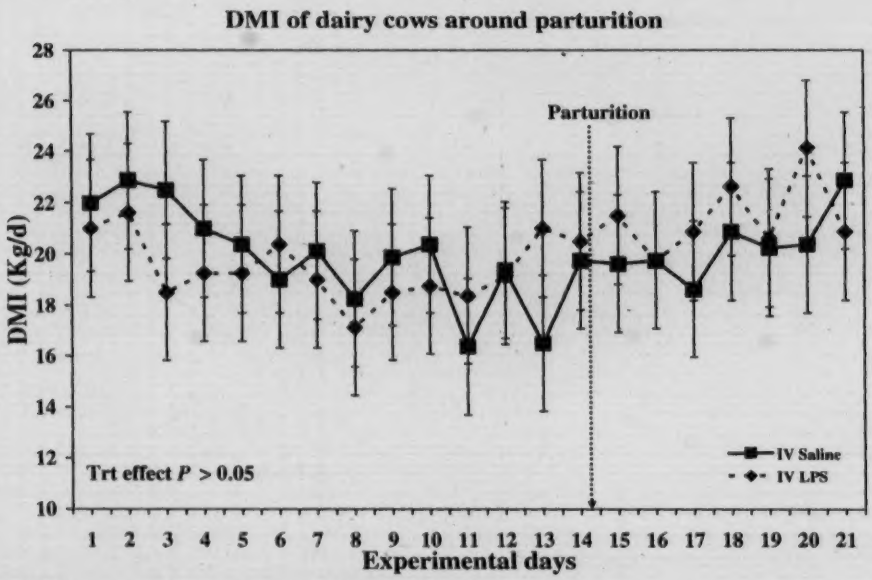

Figure 4.19. The dry matter intake of dairy cows treated intravenously with saline and LPS two weeks before and three weeks after parturition.

Table 4.1 Incidence of postpartal metabolic disorders in the two experimental groups infused intravenously with saline (control) or saline with lipopolysaccharide from *Escherichia coli* 0111:B4 (treatment) twice per wk during wk -2, and -1 before calving and 1 wk after calving.

Control Group Cow number	Clinical disease	Treatment group Cow number	Clinical disease
2413	Normal	2306	Retained placenta Metritis Ketosis
2302	Normal	2285	Retained placenta
2403	Retained Placenta Laminitis	2418	LDA Ketosis Off feed
2267	Normal	2419	Normal
2416	Normal	2167	Off feed Sickness LDA
2415	Normal	2284	Retained placenta
2363	Normal	2422	Retained placenta
9818	Normal	2366	Mastitis (4 d postpartum)

Table 4.2. The differences in various parameters in cows administered either LPS or saline intravenously beginning 2 weeks and one week before parturition and one week after parturition.

Variables	Differences recorded between the LPS treated group and control group
I. Acute phase proteins	
1. Serum amyloid A	No significant difference
2. Lipopolysaccharide binding protein	No significant difference
3. Haptoglobin	No significant difference
II. Plasma immunoglobulins	
1. Immunoglobulin A	No significant difference
2. Immunoglobulin G	Higher concentration in treatment group
3. Immunoglobulin M	Lower concentration in treatment group
III. Plasma metabolites	
1. Non-esterified Fatty acids	No significant difference
2. Beta-hydroxy butyric acid	No significant difference
3. Glucose	No significant difference
4. Lactate	No significant difference
5. Insulin	No significant difference
6. Cholesterol	Lower concentration in treatment group
7. Cortisol	Higher concentration in treatment group
IV. Plasma minerals	
1. Calcium	No significant difference
2. Iron	No significant difference
3. Zinc	No significant difference
4. Copper	No significant difference
V. Rectal temperature	No significant difference
VI. Dry matter intake	No significant difference

Chapter 5

Summary

Transition period, including 3 weeks before and 3 weeks after parturition, is the most critical period for the health of dairy cows. During the first month after calving dairy cows are often affected by one or a series of multiple metabolic and infectious diseases including acidosis, laminitis, displaced abomasum, fatty liver, ketosis, milk fever, downer cow syndrome, retained placenta, infertility, bloat, liver abscesses, mastitis, unexplained fever, and metritis. Several observational studies have indicated that metabolic diseases are interconnected with each other suggesting a common causal agent (Erb and Grohn, 1988; Correa et al., 1993); however, the search for this common agent continues and the mechanisms are still unclear.

Currently, there are two lines of thinking regarding the etiology of metabolic diseases in dairy cows. One group of scholars has suggested that metabolic diseases are related to a negative energy balance that develops immediately after calving when cows start producing high amounts of milk and feed intake drops by almost 30% of the ration dry matter (Grummer, 1993; Goff and Horst, 1997; Drackley, 1999). These groups of researchers and many others have endorsed the hypothesis that feeding transition cows a balanced diet should prevent the occurrence of metabolic diseases. They have conducted numerous investigations during the last three decades aiming at offering cows an optimal ration that should provide all the necessary ingredients to prevent the occurrence of metabolic diseases. Unfortunately, the 'perfect' diet has not been formulated yet and

currently one in two dairy cows still continues to be affected by one or multiple metabolic diseases with tremendous financial losses for dairy producers (Drackley and Dann, 2005; Ferguson, 2001).

On the other hand, a new line of thinking is emerging stressing the importance of dietary ingredients such as high starch or high fat diets in development of bacterial imbalances. Thus, the main postulate of these groups of investigators is that non-natural dietary interventions perturb equilibrium of mucosal bacterial populations resulting in release of toxic compounds (Andersen, 2003; Ametaj et al., 2005; Emmanuel et al., 2007; Emmanuel et al., 2008). The latter impair mucosal barrier functions and initiate a cascade of reactions reflected as metabolic and immune disorders and displayed clinically as metabolic and infectious diseases (Emmanuel et al., 2007; Emmanuel et al., 2008). In a nutshell, this new paradigm holds that bacterial imbalances associated with dominance of Gram-negative bacteria are associated with the release of a large amount of endotoxin (Emmanuel et al., 2008). Endotoxin, a structural component of the outer cell wall of all Gram-negatives, is able to permeate mucosal layers and increase gastrointestinal permeability to non-nutrients including pathogenic bacteria (Emmanuel et al., 2007). Translocation of endotoxin into the host blood or lymphatic circulation is associated with impaired health and multiple metabolic, immune, and production perturbations in animals resulting in sub clinical or clinical acute or chronic diseases known as endotoxin-related diseases (Andersen, 2003; Ametaj et al., 2005).

Based upon the latter line of thinking and expanding on it, we postulated that if translocation of endotoxin is at the root of multiple metabolic diseases in post parturient dairy cows; then, prevention of endotoxin transfer from gastrointestinal tract or other mucosal layers into the host systemic circulation might prevent occurrence of endotoxin-related disturbances in transition dairy cows. Repeated parenteral exposure to bacterial endotoxin is known to be associated with induction of tolerance to endotoxin. However, the strategy of parenteral exposure has been to develop humoral immune responses to endotoxin able to remove the already translocated endotoxin from host blood circulation. On the other hand, prevention of endotoxin entrance into the host system would be a better approach to prevent deleterious effects of endotoxin. To our best knowledge, no studies related to prevention of endotoxin translocation from mucosal layers into bloodstream have been conducted in dairy cattle. Therefore, the two main objectives of this research work were: to investigate the effects of repeated oral or intravenous administration of lipopolysaccharide (LPS) on different plasma metabolites and several indices of acute phase response as well as on plasma minerals, and health status of periparturient dairy cows.

5.1. Experiment 1

The objective of the first experiment was to evaluate whether repeated oral application of LPS, in increasing doses, before and after parturition would improve immune responses against endotoxin and prevent deleterious effects of endotoxin translocation in dairy cows. This experiment included sixteen pregnant multiparous and primiparous Holstein dairy cows divided into 2 treatment groups of 8 cows each, 2 wk before the expected day

of parturition. Cows were administered orally, twice per week, for 3 consecutive weeks starting 2 weeks before parturition the following treatments: 3 mL of saline (control), or 3 mL of saline containing LPS from *E. coli* 0111:B4. The amount of LPS administered each week was 0.01, 0.05, or 0.1 μg/kg BW (~650 kg cow). Blood samples were obtained from the jugular vein twice per week for 3 consecutive weeks (2 weeks before and 1 week after parturition) 15 minutes before administration of the treatment as well as once per week during week 2, 3, and 4 after parturition. Three acute phase proteins (serum amyloid A (SAA), lipopolysaccharide-binding protein (LBP), and haptoglobin (Hp) and three anti-LPS IgA, IgG, and IgM were measured to evaluate immune status of the animals. A variety of metabolites representing energy, lipid, and mineral metabolisms were measured such as NEFA, BHBA, glucose, lactate, insulin, cholesterol, Ca^{2+}, Fe^{2+}, Zn^{2+}, and Cu^{2+}. Clinical variables such as rectal temperature, respiration, rate, rumen contractions and occurrence of metabolic diseases were obtained during 2 weeks before and up to 8 weeks after parturition.

The most important finding of the first experiment was that the incidence of metabolic disorders was lower in cows treated orally with LPS. In fact, different authors have suggested a role for endotoxin in the etiology and pathogenesis of multiple postpartal diseases such as milk fever (Aiumlamai et al., 1992), laminitis (Boosman et al. 1992), fatty liver (Ametaj et al., 2008), retained placenta and endometritis (Dohmen et al., 2000), displaced abomasum (Kaze et al., 2004), acidosis (Nagaraja and Titgemeyer, 2007), and mastitis (Kauf et al., 2007). A plausible mechanism that might explain the lowering of the occurrence of metabolic diseases in our experimental cows is related to

induction of mucosal anti-LPS antibodies by repeated oral treatment with LPS and as a result prevention of endotoxin translocation and its deleterious effects. This was in agreement with our expectation and supports the postulate that endotoxin is involved in the etiology and pathogenesis of multiple metabolic and infectious diseases in transition dairy cows.

No differences in acute phase proteins (SAA, LBP and haptoglobin) between the two experimental groups were obtained. In fact, these three acute phase proteins increased in both groups around calving. Proinflammatory cytokines such as IL-1, IL-6, and TNF-alpha are the main stimulators of production of acute phase proteins by liver hepatocytes. The source of cytokines in our cows might be the mammary gland or uterine infections postpartum (Hirvonen et al., 1999). Most of the cows affected by subacute mastitis or uterine infections do not show clear clinical signs of disease. Moreover, Mackiewicz et al., (1991) and Kucharski et al. (2008) showed that proinflammatory cytokines are absorbed from infected uteri into the systemic circulation and SAA, haptoglobin, and LBP are increased during mastitis (Suojala et al., 2008). Therefore, high plasma SAA, LBP, and haptoglobin on both groups of cows after calving might be related to Gram-negative uterine and mammary gland infections. In support of this hypothesis are reports indicating that the oral presentation of antigens does not protect the mucosa of the reproductive tract of women from infection by *Vibrio cholerae* (Kozlowski et al., 1997).

Another finding of this research was that plasma ant-LPS IgA was not different between the two experimental groups whereas anti-LPS IgG was lower and anti-LPS IgM was

greater in cows treated with oral LPS. It is speculated that the lack of plasma IgA response in treated cows might be related to the fact that oral administration of antigens stimulates more mucosal secretion of IgA instead of the systemic one. Indeed, lack of plasma IgA responses were reported in mice treated orally with LPS to prevent sepsis induced by cecal ligation and puncture (Márquez-Velasco et al., 2007). Lower anti-LPS IgG antibodies in cows treated orally with LPS is a positive response indicating prevention of LPS translocation into circulation. This might be related to the phenomenon of oral tolerance, in which oral immunization with an antigen results in the development of suppressor T cells which inhibit IgG responses (Márquez-Velasco et al., 2007). Greater anti-LPS IgM antibodies in treated cows also indicate that oral administration of LPS modulated humoral immune responses favoring protection against endotoxin. These data are in agreement with previous research indicating that pretreatment of mice with oral LPS increased plasma anti-LPS IgM and protected them from sepsis induced by cecal ligation and puncture (Márquez-Velasco et al., 2007).

Plasma cortisol, another hormone released by HPA axis during conditions of endotoxemia, also did not differ between the two treatment groups. The HPA axis is highly activated during endotoxemia as evidenced by elevated plasma ACTH levels, increased cortisol secretion, and elevated serum total and free cortisol levels (Hamrahian et al., 2004; Arafah, 2006; Ho et al., 2006). In addition to increased adrenal production or secretion of glucocorticoids during endotoxemia, impaired glucocorticoid clearance can contribute to the greatly increased serum cortisol concentrations (Melby and Spink, 1958). Moreover, during endotoxemia proinflammatory cytokines IL-1, IL-6, and TNF-

alpha stimulate secretion of glucocorticoids (including cortisol) from adrenal gland. The lack of differences in plasma cortisol levels between the two groups of cows suggest that no activation of HPA axis occurred.

The better clinical outcome of the oral-LPS treated cows was supported by the observation that the concentrations of NEFA and BHBA in plasma changed only slightly and remained within normal ranges in cows treated with oral LPS. This was not true for control cows which had typically high plasma NEFA and BHBA immediately after parturition. Furthermore, plasma glucose, lactate, and insulin were greater in LPS-treated cows. No effect of treatment was obtained for plasma cholesterol. The beneficial effects of the oral administration of LPS with regards to improved concentrations of plasma metabolites that are associated with fatty liver (i.e., NEFA) and ketosis (i.e., BHBA) and general improvement of energy balance as reflected by greater plasma glucose and insulin, further demonstrates and support the hypothesis that endotoxin is involved in the etiology of metabolic disturbances during the transition period. Intravenous infusion of LPS has been associated with disturbances of glucose, NEFA, BHBA, and insulin in plasma of dairy cows (Steiger et al., 1999; Waldron et al., 2003). Therefore, prevention of endotoxin translocation into the blood circulation by oral immunization with LPS might have contributed to a better energy balance in the treated cows in our experiment by preventing translocation of endotoxin and its harmful effects.

Additionally, plasma Ca^{2+} and Fe^{2+} did not differ between the control and treated cows; however, plasma Zn^{2+} increased whereas plasma Cu^{2+} decreased in cows treated with oral

LPS. The decrease in plasma Ca^{2+} observed in both groups immediately after calving might be related to the large amounts of acidogenic (i.e., rich in starch) diets fed to cows immediately after parturition. Although not significant, a decrease in plasma Fe^{2+} was observed in treated cows. It is known that the host responds to presence of LPS with withdrawal of plasma Fe^{2+}. Therefore, exposure of cows to oral LPS might have affected Fe^{2+} availability. Interestingly, both plasma Zn^{2+} and Cu^{2+} were affected by the oral treatment with LPS. In fact, plasma Zn^{2+} usually withdraws during inflammatory conditions and mounting of an acute phase response. Therefore, enhanced plasma Zn^{2+} might be seen as a positive response of treated cows indicating the lack of an inflammatory response in those cows. In support of this postulate are also data related to plasma Cu^{2+}. Generally, plasma Cu^{2+} increases during inflammatory conditions (Kushner, 1982; Shenkin, 1995). Copper is an essential element for normal functioning of a series of antioxidant enzymes indispensable to oppose side effects of endotoxin. Lower plasma Cu^{2+} in treated cows supports the positive clinical outcome of treated cows and the lack of an inflammatory state in those cows.

Finally, no differences were obtained in DMI between the two groups. Indeed, our data are in line with other reports that oral administration of LPS has no effect on feed intake in ruminants or other livestock animals (Cort et al., 1990; Holst et al., 1993). For endotoxin to have an effect on feed intake it should translocate into the systemic circulation and stimulate the production of IL-1, IL-6 and TNF-alpha. The latter are known to have a direct inhibitory effect on hunger center in the hypothalamus (Buchanan

and Johnson, 2007). Our data again support our postulate that oral administration of LPS has prevented translocation of LPS into the systemic circulation.

Taken together results of the first experiment indicated that repeated oral administration of LPS from *Escherichia coli* 0111:B4 during peripartum improved immune status and prevented clinical and metabolite disturbances typically observed in dairy cows around parturition. This suggests a causal role for endotoxin in the etiology and pathogenesis of metabolic disorders in dairy cows and the potential utilization of oral treatment against LPS for prevention of endotoxin translocation and its harmful effects in periparturient dairy cows.

5.2. Experiment 2

The objective of the second experiment was to evaluate whether repeated intravenous administration of LPS during the peripartum would render the transition dairy cows more resistant to postpartal endotoxin-related metabolic and infectious diseases. This experiment involved sixteen pregnant Holstein cows divided into 2 groups of 8 cows each to be infused twice per week intravenously with either 100 mL of saline or three increasing dosages of LPS from *Escherichia coli* 0111:B4 at 0.01, 0.05, and 0.5 μg/kg BW dissolved in 100 mL of saline during -2 and -1 week before the anticipated day of parturition as well as during the first week after calving. Blood samples taken from the jugular vein were analyzed for the same variables described in the first experiment. In addition, rectal temperature, respiration rate, rumen contraction, and dry matter intake (DMI) were measured.

Results of this experiment indicated that intermittent experimental endotoxemia was associated with increased incidence of metabolic and infectious diseases in cows infused with intravenous LPS. These data support the line of thinking that postulates a role for endotoxin on the etiology and pathogenesis of multiple metabolic diseases in transition dairy cows. Moreover, our data are in disagreement with research suggesting utilization of intravenous LPS for induction of tolerance to endotoxin and prevention of endotoxin-related diseases in animals.

Results of this experiment also indicated that intermittent endotoxemia was associated with enhanced plasma anti-LPS IgG antibodies and decreased plasma anti-LPS IgM antibodies in the treated cows. It is known that humoral immune response is divided into the primary (i.e., IgM) and secondary (i.e., IgG) immune responses based on the number of times that the host is exposed to an antigen. During the first encounter to an antigen an IgM antibody response develops; however, reappearance of the same antigen induces an IgG immune response (van Rooijen, 1987). In our case, the LPS was administered to cows 6 times during the 3-weeks of the experimental period. Therefore, greater plasma anti-LPS IgG antibodies in the treated cows are indicative of the development of a secondary immune response to endotoxin.

Cows treated with LPS also had greater plasma cortisol concentrations compared to control cows. There is a bi-directional communication between the neuroendocrine and immune systems that maintains or restores homeostasis during inflammatory conditions

or endotoxemia. Exposure to endotoxin, for example, elicits the release a variety of pro-inflammatory cytokines that activate the HPA axis (You et al., 2008). The secretion of cortisol plays a significant role in down regulation of the host inflammatory response, minimizing potential tissue damage. Therefore, greater plasma cortisol in cows infused intravenously with LPS is a protective response to the presence of endotoxin in plasma.

Cows administered LPS in blood also had lower plasma cholesterol compared to those of the control group. Our results are in agreement with previous research that has shown that administration of LPS is associated with a decrease in plasma cholesterol (Hardardottir, 1994). For example, other research demonstrated that administration of LPS suppresses lecithin:cholesterol acyltransferase (LCAT) mRNA levels in the liver and LCAT activity in the plasma. Lecithin:cholesterol acyltransferase is an enzyme produced by the liver that converts free cholesterol into cholesteryl ester (a more hydrophobic form of cholesterol) which is then sequestered into the core of a lipoprotein particle (Dobiásová and Frohlich, 1999). The enzyme is bound to high-density and low-density lipoproteins in the blood plasma. Suppression of LCAT expression and activity by infused LPS might have caused lower plasma cholesterol in LPS-treated cows.

Results also showed that no differences between the two groups were recorded in relation with: 1) innate and acquired immune responses (i.e., plasma SAA, LBP, haptoglobin, and anti-LPS IgA antibodies), 2) metabolic responses related to energy and lipid metabolism (i.e., NEFA, BHBA, glucose, lactate, and insulin), 3) mineral responses (i.e., plasma Ca^{2+}, Fe^{2+}, Zn^{2+}, and Cu^{2+}), and 4) clinical responses (i.e., rectal temperature, respiration

rate, rumen contraction, and DMI). It has long been recognized that intravenous administration of bacterial endotoxin into animals or humans results in increasing resistance to its toxic effects (Greisman and Woodward, 1970). This phenomenon is known as tolerance to endotoxin. During endotoxemia, the host responds to LPS with a systemic production of proinflammatory cytokines and a variety of metabolites, which aim at eliminating the invading pathogens and restore homeostasis (Shahin et al., 1987). Although the cytokines and metabolite responses are indispensable for prevention of pathogen growth and dissemination (Hagberg et al., 1984; Eden et al., 1988; Cross et al., 1995), an excessive and chronic response is potentially auto destructive and may lead to metabolic dysfunctions, tissue damage, septic shock, and eventually death of the host (Beutler et al., 1985, Galanos and Freudenberg, 1993). The phenomenon of endotoxin tolerance is known from animal models of "sterile infection" induced by LPS: after an initial low dose of LPS, animals are protected against the detrimental consequences of a subsequent high dose of LPS. This protection is associated with an attenuated response to LPS (Erroi et al., 1993) due to a down-regulation of immune and metabolic responsiveness (Fahmi and Chaby, 1993). Results of our study demonstrated that repeated intravenous exposure to LPS induced immune, metabolic, mineral, and clinical tolerance to endotoxin in periparturient dairy cows.

In conclusion, repeated exposure to LPS was associated with high incidence of postpartal diseases in transition dairy cows and modulation of a limited number of immune and metabolic variables. Most of the immune, metabolic, and clinical variables measured did not change between the two groups of cows suggesting development of tolerance to

endotoxin. Our data do not support utilization of multiple intravenous administrations of LPS as a treatment for prevention of endotoxin-related diseases in periparturient dairy cows.

Overall, repeated oral exposure to LPS prevented occurrence of postpartal metabolic and infectious diseases related to endotoxin and improved blood metabolic and immunologic picture of periparturient dairy cows. Our data support utilization of oral administration of LPS as a preventive tool for multiple metabolic and infectious diseases related to Gram-negative bacteria and endotoxin in periparturient dairy cows. Results also showed that intermittent endotoxemia, induced by repeated intravenous administration of increasing doses of LPS, enhanced occurrence of endotoxin-related diseases in periparturient dairy cows and modulated humoral immune responses, cortisol, and cholesterol. Additionally, repeated intravenous administration of LPS was associated with immune, metabolic, and clinical tolerance to endotoxin in experimental animal. Because intravenous LPS was unable to confer protection against endotoxin-related diseases in transition dairy cows we do not recommended its utilization as a preventive strategy against endotoxin-related diseases in dairy cows. Finally, there was an interesting observation regarding the incidence of metabolic diseases in the control groups of cows between the first and the second experiment with the latter having greater incidence of those diseases. The reason for this discrepancy is not known and warrant further research with a greater number of animals per experimental group.

5.3. Literature cited

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